IN THE UNITED STATES PATENT AND TRADEMARK OFFICE BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application of

Applicants	:	Catherine M. Verfaillie and Yuehua Jiang
Application No.	:	10/561,826
Filed	:	October 17, 2006
Title	:	Neuronal Differentiation of Stem Cells
Examiner	:	Chang Yu Wang
Attorney Docket No.	:	890003-2006.1
Art Unit	:	1649

Cleveland OH 44114

Date: May 23, 2011

MAIL STOP APPEAL BRIEF- PATENTS
Commissioner for Patents
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APPELLANTS' BRIEF ON APPEAL UNDER 37 C.F.R. § 41.37

Sir:

This is an Appeal filed in response to the Final Office Action, dated June 22, 2010, and a Notice of Appeal filed October 22, 2010.

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Real Party In Interest

The real party in interest is ABT Holding Company by virtue of an exclusive license from the Regents of the University of Minnesota, the Assignee of the application by means of the Assignment recorded at Reel/Frame 014629/0318.

Related Appeals and Interferences

There are no other prior or pending appeals, interferences or judicial proceedings known to Appellants, to Appellants' legal representative or to the real party in interest which may be related to, directly affect, or be directly affected by or have a bearing on the Board's decision in the pending appeal.

Status of Claims

Claims 1, 5, 6, and 13 are pending in the present application. Claims 2-4 and 7-12 are canceled. The following claims stand or fall with claim 1: 5, 6, and 13.

Claims 1, 2, 5-9, 11, and 13 were finally rejected under 35 U.S.C. § 103(a) on the grounds that they are unpatentable over Studer (WO 02/086073) ("Studer") in view of Lee (U.S. 2003/0211605) ("Lee") in the Office Action mailed June 22, 2010.

Claims 1, 2, 5-11, and 13 were finally rejected under 35 U.S.C. § 103 on the grounds that they are unpatentable over Studer in view of Lee and further in view of Song (*Methods in Molecular Biology*, 198:79-88 (2002)) ("Song") in the Office Action mailed June 22, 2010.

The rejection of claims 1, 5, 6 and 13 is being appealed.

Status of Amendments

Appellants file an Amendment to previously pending claims 1, 2, and 5-13, subsequent to the Final Office Action dated June 22, 2010, by canceling claims 2 and 7-12. The claims as they stand on Appeal are contained in the Claims Appendix to this Brief.

Summary of Claimed Subject Matter

Independent claim 1 is directed to methods for inducing stem cells to differentiate into neuronal cells by performing a four-step sequential protocol wherein step a) involves culturing the starting cells with basic fibroblast growth factor (bFGF) so that the cells adopt a specific phenotype. In step b), the cells with the phenotype produced by step a) are cultured with fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) to produce cells with another phenotype. In step c), the cells with the phenotype produced by step b) are then exposed to brain derived neurotrophic factor (BDNF) to produce cells with another phenotype. In step d), the cells with the phenotype produced by step c) are then co-cultured for seven days with astrocytes. All steps are performed for at least seven days.

Support

Support for all the limitations in this claim can be found in Appellants' specification *inter alia* on page 3, lines 23-30, and page 4, lines 3-4. Support is also found in the original claims 1 and 4.

Grounds of Rejection to be Reviewed on Appeal

Whether claims 1, 5, 6, and 13 are unpatentable over Studer and Lee under 35 U.S.C. § 103(a).

Whether claims 1, 5, 6, and 13 are unpatentable over Studer and Lee and further in view of Song under 35 U.S.C. § 103(a).

Argument

CLAIMS 1, 5, 6 -AND 13 ARE NOT OBVIOUS OVER STUDER OR LEE, ALONE OR IN COMBINATION

What Studer and Lee Disclose

First, Appellants point out that both of the references teach the same basic differentiation procedure. (Lorenz Studer is named on both documents.) The difference is that Studer (WO 02/086073) starts with nuclear transfer embryonic stem cells and Lee (U.S. 2003/0211605) starts with blastocyst-derived embryonic stem cells. Differentiation procedures are individually designed to produce, as the end product, dopaminergic neurons, serotonergic neurons, astrocytes, oligodendrocytes, or GABA-ergic neurons.

The differentiation protocols in these references involve five distinct stages, which Studer and Lee refer to as stages I-V. At each stage the cells from the previous stage are transferred to new containers. See any of Figure 5 and page 4 of Studer and Figure 1, paragraph 46, and paragraphs 12-17, of Lee for the basic steps.

In stage I, the embryonic stem cells (ESC) are allowed to form an embryoid body. In stage II, the resulting embryoid body is placed in a new container with an attachment factor. In stage III, the embryoid body is again transferred to a new container and allowed to grow for nine to sixteen days, after which time the embryoid body contains cells that express the neural stem cell marker nestin. The reference refers to this as "nectin." The expression of nestin signals "commitment" to differentiate into cells of neural fate. The cells formed in stage III, therefore, are no longer ES cells. None of these stages requires any of the claimed factors.

In stage IV, a "specification" protocol is applied that primes the cells for differentiation in stage V. In stage IV, the committed nestin-positive cells are exposed to a mitogen and, except for the protocol to

generate GABA-ergic neurons, also exposed to FGF8 and SHH. The mitogen often is bFGF. In stage V, the cells of stage IV are differentiated by mitogen withdrawal.

Lee (which predates Studer) and Studer disclose protocols that maximize production of dopaminergic neurons. The references also indicate that, with this procedure, the end product (i.e., at the end of stage V) contains some serotonergic neurons. See Studer, paragraph 59 and Lee, paragraphs 224-226. Studer, on the other hand, also discloses specific modifications of the protocol to produce GABA-ergic neurons, astrocytes, oligodendrocytes, and a higher number of serotonergic neurons.

These references are complex and may disclose many different modifications of the basic protocol. For the convenience of the Board, Appellants provide a summary schematic representation of each of the disclosed protocols from Studer and Lee. See Evidence Appendix (1). Appellants have numbered these protocols for reference. The numbers are in the first column. The second column indicates the paragraph in Studer or Lee wherein that numbered protocol is disclosed. The arrows represent the individual stages in the five-stage protocol. The numbers above the arrows designate the stage of the protocol.

To illustrate, using, as an example, Studer's disclosure of protocols to generate dopaminergic neurons, see number 14 in the section showing Studer's protocols. The protocol is found in paragraph 15 of Studer. It is the five-stage procedure. In stage IV, the mitogen is bFGF. SHH and FGF8 are added with the bFGF to cause an increase in the specification of dopaminergic neurons over serotonergic neurons. So, adding these two factors increases the ratio of dopaminergic to serotonergic neurons.

Thus, both of these references disclose discrete protocols for generating specific end products. The basic idea, however, is to practice the first three steps without any of the factors, to add a mitogen at the fourth step, and to withdraw the mitogen at the fifth step.

Examiner's Arguments

Claims 1, 5, 6, and 13 are rejected under 35 U.S.C. § 103(a) on the grounds that they are obvious over Studer in view of Lee. The Examiner states that the rejection is maintained "for the reasons of record." The reasons of record, however, are not necessarily consistent from one Office Action to the next. Accordingly, Appellants present below what they believe to be the reasons of record.

Office Action dated March 17, 2008

Here the Examiner's rationale appeared to have supported a rejection on the grounds of anticipation. Regarding Studer, the rationale for rejecting claims 1 and 13 was that Studer "teaches a method of inducing stem cells to differentiate into neuronal cells comprising culturing embryonic stem cells in the presence of bFGF, FGF8, SHH, BDNF, and co-culturing the cells with astrocytes as recited in instant claims 1 and 13." Page 7 of the Office Action. There is no discussion in the Office Action about how the Studer protocol differs from the claimed protocol and why it would have been obvious to alter the Studer protocol to arrive at the method of claims 1 and 13. And, the Examiner appears to have focused on the final step (co-culturing the cells with astrocytes), as she repeats that Studer "teaches differentiation of cultured embryonic stem cells into neurons and astrocytes, which meets the limitation of 'comprising co-culture astrocytes' [sic] as recited in instant claims 1 and 13..." Page 7 of the Office Action.

The Examiner also addressed (original) claim 4, which recited that each of the steps was performed for at least seven days. The Examiner's rationale was that "each step and each stage of the culture conditions of [Studer] require six to nine days and the whole culture procedures [sic] take more than a month...which is within the limitation of the instant claim 4." Page 8 of the Office Action.

The Examiner relied on the same rationale to reject claims 1 and 13 over Lee. Page 8 of the Office Action.

Thus, there was no explicit rationale in the Examiner's discussion to explain why the protocol of claims 1 and 13 would have been obvious over the references, either singly or in combination. The Examiner's rationale supported a rejection on the grounds of anticipation.

Final Office Action dated December 24, 2008

The Examiner's rationale appears to be the following: "Although the instant method recites adding bFGF, FGF8, SHH, BDNF [sic] sequentially, *at the end of the final steps, the culture medium contains the identical growth factors as those in Studer's* to induce neuronal differentiation." Page 7 of the Office Action. Italics added. Appellants understood the Examiner's point to be the following: Sequential addition would inherently encompass adding the factors within a matter of seconds or minutes or even hours (i.e., before they have a chance to affect the cells and alter the phenotype). It would have been obvious to add all the factors one at a time at the beginning of the procedure.

(Appellants could see the Examiner's logic as it pertained to claims 1 and 13, which did not recite the time period of seven days for each step. Accordingly, Appellants incorporated (into claims 1 and 13) the limitations of claim 4, that each step is at least seven days.)

The Examiner also maintained the position that the Studer end products encompass neurons and astrocytes and, thus, meet the limitation of co-culturing the neuronal cells with astrocytes. Page 8 of the Office Action.

Office Action dated October 29, 2009

The essential rationale is found on page 6 of the Office Action as follows.

> [T]he claimed method is directed to inducing neuronal differentiation using the same growth factors (bFGF, FGF8, SHH, BDNF) and the same ES cells, which are taught by the cited references. Although the claimed method alters the way of adding growth factors, and phenotypical cell types may have different proportions during the recited

culturing procedures, at the end of the steps, the end result of neuronal differentiation is expected and [sic] to generate dopaminergic neurons.

On page 10 of the Office Action, the Examiner states, "taken together the claimed method of sequentially adding the factors to ES cells is obvious over the cited references because Lee and Studer do provide a motivation and an expectation of success to add growth factors sequentially."

The Examiner provided no evidentiary support that modifying the Studer (or Lee) protocol to the claimed protocol would, indeed, have been expected to generate dopaminergic neurons.

Appellants' Reply

Neither Studer Nor Lee Anticipates the Claims

Contrary to the Examiner's argument in the first Office Action, none of the protocols in Studer or Lee is the same as the protocol of claim 1. The closest protocol is the protocol designed to produce dopaminergic neurons. It is the only protocol in which all the four claimed factors are used. But, even this procedure does not contain all of the elements of the claims. See Evidence Appendix (1), Studer protocols numbers 14-21 and Lee protocols numbers 1-13. None of the permutations is the same as in claim 1.

One Would Not Arrive at the Claimed Protocol by Combining the References

Nor do the combined references produce a method with all of the elements of claim 1. Evidence Appendix (1) schematically demonstrates each of the recommended protocols that produce desired end products. Studer discloses specific discrete protocols for dopaminergic neurons, serotonergic neurons, GABA-ergic neurons, oligodendrocytes, and astrocytes. Lee, however, only discloses procedures designed to produce dopaminergic neurons (although it appears that, in this procedure, there is small number of serotonergic neurons that are also inherently produced as an end product). See Lee, paragraphs

224-226. The only procedure in which all of the four claimed factors are used is in the protocol for generating dopaminergic neurons. But, even this procedure does not contain all of the elements of the claims. See Studer protocols numbers 14-21 and Lee protocols numbers 1-13.

"Co-culture with Astrocytes"

There are three types of disclosure in these references that explicitly relate to astrocytes. But none of these disclosures meet the limitation of step d), i.e., co-culturing with astrocytes. The three disclosures are discussed below.

(1) Some glial cells are differentiated along with dopaminergic neurons in stage V

Studer indicates that some glial cells are produced as an end product in the procedure for generating dopaminergic neurons as the end product. See Studer, paragraph 76. Studer does not characterize the nature of the glial cells that are produced. These glial cells may or may not be astrocytes. Astrocytes are a sub-type of glial cell. See Evidence Appendix (4). Lee distinguishes between astrocytes and glial cells. In paragraph 47, Lee states that non-neural cell types include astrocytes, oligodendrocytes, and glial cells.

But, even if one assumes, for argument's sake, that the glial cells are astrocytes, there is no teaching that any cell is incubated with an astrocyte for the seven day time period. Any astrocyte would be an end product of the protocol, produced by the stage V conditions. So, if the references did indeed meet this limitation (seven-day co-culture), the astrocytes would have to be produced immediately (i.e., at the beginning of stage V) or they could not be co-incubated for seven days. And there is no indication in the reference that this occurs. It is just as likely that it takes the entire time during which stage V is conducted to generate, finally at the end, astrocytes and neurons.

(2) Protocol specifically designed to generate astrocytes

The second type of disclosure, found in Studer, is a differentiation protocol specifically designed to produce astrocytes. Studer does not discuss whether, in this particular procedure, any neuronal cells are produced as an end product along with the astrocyte end product (i.e., at the end of stage V). However, even if some neuronal cells are produced, there is no teaching that the neuronal end product is incubated with an astrocyte end product for the seven day time period recited in the claims. If the references did indeed meet this limitation, the astrocytes and the neurons would have to be produced immediately (at the beginning of stage V) or they could not be co-incubated for seven days. And there is no indication in the reference that this occurs. It is just as likely that it takes the entire time during which stage V is conducted to generate, finally at the end, astrocytes and neurons.

(3) Cell culture containing dopaminergic neurons and astrocytes

There is disclosure in Lee that refers to a cell culture in which there are both neurons and astrocytes. See Lee, paragraphs 21, 42, and 147. This, however, appears to be the end product that is produced at the end of stage V. There is no disclosure that the actual end products are cultured together, according to step d) of the claims, for seven days.

Therefore, contrary to the Examiner's assertions, there is no disclosure in Lee or Studer to practice step d).

Accordingly, even if one were to combine the references, the claimed invention would not be obtained.

No Reasonable Expectation of Success/Lack of Motivation

Appellants assume, for argument's sake (although this has not been tested), that adding the four factors to the nestin-positive cells (in stage III) sequentially would produce the same result as simultaneous exposure if one were to add them all *within minutes, or perhaps even hours,* of each other. But, where each individual step is carried out for *at least seven days,* one would not have expected the same result as simultaneous exposure.

Each factor induces a specific phenotypic effect. If the factors are all added simultaneously, it is not reasonably predictable that the phenotype will be the same as the phenotype produced by exposing the cell to the factors sequentially for the seven day period. Accordingly, performing the method sequentially, with at least seven days for each step, would not have reasonably predicted cells with the same phenotype. More likely than not, the cells should be different.

In the cited art all factors are exposed to the same initial cell type (a neural-committed cell). But in the claimed method each factor acts on four phenotypically discrete cell types, i.e., the FGF8 and SHH act on the specific and discrete cell produced in step (a) by bFGF, the BDNF acts at the specific and discrete cell produced in step (b) by FGF8 and SHH, and the astrocytes act on the specific and discrete cell produced in step (c) by BDNF. Because one would expect four different phenotypic cell types, it is not reasonably predictable that the final cell type will be the same cell as produced by the prior art methods. Accordingly, the person of ordinary skill would not have been motivated to modify the procedure as Appellants have done because they would not have known what to expect; the results were not reasonably predictable.

Appellants presented the attached Declaration of Dr. Catherine Verfaillie, an expert in the field of stem cell research, and one of the inventors in the above-captioned application. Dr. Verfaillie explained the Appellants' position discussed above with more particularity and detail. She explained that the rejection is based on an incorrect scientific reasoning about how the cells would respond to each of the claimed

factors when they are administered simultaneously as opposed to when they are administered sequentially. She also explained how sequential administration creates four discrete cell types that would be phenotypically unique and would be expected to have a unique response to the growth factor to which it is exposed. She explained that the end product of sequential exposure cannot be reasonably predicted based on the results of simultaneous exposure to the same factors. This explanation is found starting on page 2 of the Declaration. She also referred to an example of this principle in a reference (Snykers et al.) attached to the Declaration. She concluded that, in her opinion as an expert in the field, qualified to speak to what the person of ordinary skill in the art would have expected, this hypothetical person would not have been motivated to drastically alter the procedure of Studer and Lee as in the method as now claimed because they would not have reasonably expected to produce the same results.

Based on the Declaration and scientific reasoning by Dr. Verfaillie, Appellants submit that the person of ordinary skill would not have been motivated to modify the procedure to that of claim 1 because it was not reasonable to expect the same end product. After all, Studer and Lee designed discrete protocols for producing a specific desired end product. In paragraph 85, Studer itself explicitly discloses that "the composition of the medium is crucial for determining the type of CNS cell that will be generated." This shows that the specific protocol has been tailored to produce the desired end product.

So, even if one had been motivated "to try" this approach, they would not have reasonably expected that it would produce the same result (or even a good result). The result simply was not reasonably predictable; another way of stating this is that there was not a reasonable expectation of successfully producing the prior art end product.

The Examiner cited *KSR International v. Teleflex Inc.* (82 USPQ2d 1385 (US2007)). This test was not disturbed by the Supreme Court in its rationale for the decision in *KSR*. In fact, the Supreme Court emphasized that an invention may be "obvious to try," but to support a *prima facie* case of obviousness, "reasonable predictability" is required.

The Examiner believes that with sequential addition the cell eventually is exposed to all factors and so one would expect the same end result. But, as Dr. Verfaillie has explained, each factor induces some phenotypic change on the cell to which it is exposed. At the end, one has a cell that has discrete phenotypic predecessors: a first predecessor not expressing nestin; a second predecessor produced by inducing a new phenotype with bFGF after bFGF; a third predecessor produced by inducing a new phenotype with SHH and FGF8, and; a fourth predecessor produced by inducing a new phenotype with BDNF. By the time all factors are present, the cell, more likely than not, is different from the cell that Studer and Lee produced. Therefore, it is not reasonable to expect the same end product if one uses a sequential approach as Applicants seek to claim, instead of a simultaneous ("cocktail") approach.

Nevertheless, the Examiner maintains the rejection for the reasons of record and erroneously dismisses the Declaration of Dr. Verfaillie. Moreover, the Examiner provides no scientific evidence to rebut the statements of Dr. Verfaillie regarding the differences of these approaches.

The Examiner's rationale for dismissing Appellants' Declaration is that (1) the Declaration does not provide a side-by-side comparison of the same cells and the same growth factors added simultaneously and sequentially to show that the two results are different; and (2) the evidence that Appellants provided to show that simultaneous and sequential exposure of progenitor/stem cells to differentiation factors produces two different results were not performed with the same cells as in the cited references and the same differentiation factors (i.e., differentiation of progenitor cells into neurons). For these reasons, the Examiner asserted that the Declaration was insufficient to overcome the rejection.

Side-By-Side Comparison Not Required

The Examiner dismissed Dr. Verfaillie's Declaration on the grounds that Dr. Verfaillie "fails to provide side-by-side comparisons to demonstrate that the claimed cell types, or end products generated from sequential addition of growth factors, are different from those that are simultaneously exposed to the same growth factors taught in the cited references." Page 6 of the Office Action dated October 29, 2009.

This is an erroneous reason to dismiss this Declaration. Obviousness is premised on: (1) what the person of ordinary skill would have been motivated to do; and (2) whether the person of ordinary skill would have reasonably expected that they would successfully produce the results. Thus, the opinion of Dr. Verfaillie goes to what the person of ordinary skill in the art would have expected and not to what actually occurred after the fact. A side-by-side comparison shows what actually occurs. But the proper question for obviousness is what the person of ordinary skill would have expected.

Dr. Verfaillie explained that the person of ordinary skill would have expected different results from simultaneous and sequential exposure, even to the same growth factors. Thus, having modified the procedure of the cited references as the Examiner suggests, the person of ordinary skill in the art would not have known what to expect. They would not have reasonably expected that they would successfully produce the end product (for example, dopaminergic and serotonergic neurons) as were produced by the prior art method. Thus, they might have been motivated to try some modification or other, but, again, they would not have been able to reasonably expect a successful end result.

Snykers Reference Illustrates Relevant Principle

The Declaration is also dismissed on the grounds that "the differentiated cells in the cited reference by Dr. Verfaillie are not relevant to the instant application because they are differentiated into different cells and, therefore, can't be compared with stem cell differentiated into neurons." Page 6 of the Office Action dated October 29, 2009.

The evidence submitted by Dr. Verfaillie need not be directed to the same cells. Dr. Verfaillie's evidence illustrated the principle that exposure of a progenitor cell to the same factors, but in a different sequence, will not necessarily produce the same differentiated end product. Appellants sought to establish what the person of ordinary skill in the art would have been motivated to do and what they would have reasonably expected, not what actually happens after the fact

As discussed above, the Examiner argues that the claimed method and the cited references are directed to the same goal, using the same materials (neuronal differentiation "using the same growth factors) and the same ES cells. At the end of the steps, the end result of neuronal differentiation is expected and [sic] to generate dopaminergic neurons." There is no scientific explanation about why this would have been expected. There is only this unsupported assertion. But Dr. Verfaillie's Declaration explains why it would not have been expected. The Examiner fails to present any evidence to counter the scientific reasons and conclusions of Dr. Verfaillie.

The References Teach Away

In point of fact, Lee teaches away from adding the SHH/FGF8 at a later stage than the bFGF.

Specifically, to carry out this procedure according to the directions of the inventors, bFGF, FGF8, and SHH are added together. Lee reports that when bFGF was added prior to FGF8 and SHH (i.e., earlier stage), this was ineffective to produce the dopaminergic neurons. See Lee, paragraph 205.

Lee discloses that applying the specification factors (i.e., SHH and FGF8) after expansion is ineffective. To achieve the dopaminergic neuron end product, the specification factors must be present while the cells are expanding (i.e., at the same time that the mitogen is present). Logically, one might have expected that one could produce dopaminergic neurons by applying the specification factors to cells that been expanded by the mitogen (i.e., mitogen at stage IV and specification factors at stage V). But Lee shows that this is not effective. So the reference teaches away from modifying the dopaminergic neuron protocol in this manner.

And, as discussed above, Studer has commented that the composition of the medium is critical for achieving a specific desired end product.

Accordingly, contrary to the Examiner's assertions, the person of ordinary skill in the art would have been negatively motivated to modify the Studer or Lee protocol and use the claimed protocol to produce dopaminergic neurons.

The Examiner's Numerous and Particular Citations

In the course of prosecution, the Examiner made numerous specific citations to Studer and Lee. In this Brief, with the exception of a few citations that are directed to supporting the Examiner's general conclusion, Appellants feel that it would interfere with the readability of this Brief if Appellants were to reply (in the body of the Brief) to every single citation that the Examiner has made. There are close to 50. Appellants do not, however, decline from addressing each of these. Appellants do so, for the Board's interest, in Evidence Appendix (3). Appellants believe that many of these citations are actually mis-statements and, at the very minimum, are not (in Appellants' opinion) on-point to support the Examiner's general rationale for rejecting the claims.

CLAIMS 1-11 AND 13 ARE NOT OBVIOUS OVER STUDER AND LEE AND FURTHER IN VIEW OF SONG

The Examiner does not explain how Song would apply to claims 1 and 13. Song does not cure the deficiencies of Studer or Lee.

Song prepares primary cultures of bone marrow or umbilical cord blood mononuclear cells and exposes the cultures to certain differentiation protocols (retinoic acid and EGF or BDNF). Using immunocytochemistry, Song shows that some of the cells stain positive for nestin, neuron-specific nuclear protein, and glial acidic fibrillary protein. Song does not isolate or characterize the cells that express the neural genes in response to the retinoic acid and EGF or BDNF. Song refers only to stromal cells (which are not homogeneous). In any event, Song does not disclose any protocol even similar to the claimed protocol.

Conclusion

The references, singly or combined, do not disclose the particular protocol that is claimed. The schematic diagrams in the Evidence Appendix show visually what the various modifications are. Further, the person of ordinary skill in the art would not have been motivated to modify any of the specific disclosed protocols to arrive at the claimed protocol. The person of ordinary skill in the art would not have reasonably predicted that, more likely than not, the modified protocol of the claims would result in a desired end product (i.e., dopaminergic neurons according to the references). Based on his/her knowledge of the art – as evidenced by the Verfaillie Declaration – the person of ordinary skill in the art would not have been able to reasonably predict because the results of the modification were unpredictable. The closest protocol is the one to produce dopaminergic neurons. It is the only protocol in which all four claimed factors are present. But even here the references teach away from the particular claimed procedure.

Considering all text cited by the Examiner, all guidance from the references, the Verfaillie Declaration, and Appellants' arguments, neither of the references or the references combined can be shown to suggest the claimed method of subjecting a stem cell to bFGF first for at least 7 days, then culturing the cells produced with FGF8 and SHH for at least 7 days, then culturing the cells produced in that last step with BDNF for at least 7 days, and then co-culturing the cells produced in that last step with astrocytes.

Appellants believe that fees for a five-month extension of time are due with this filing. Such payment is being made simultaneously with this filing via credit card. The Commissioner is hereby authorized to charge any deficiency in payment, or credit any overpayment, to Deposit Account No. 20-0090. The Appellants hereby authorize the Commissioner under 37 C.F.R. §1.136(a)(3) to treat any paper that is filed in this application which requires an extension of time as incorporating a request for such an extension.

Respectfully submitted,

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CLAIMS APPENDIX

1. (Previously Presented) A method for inducing stem cells to differentiate into neuronal cells comprising:

a) culturing said stem cells with basic fibroblast growth factor;

b) culturing the cells of step a) with fibroblast growth factor 8 and Sonic Hedgehog;

c) culturing the cells of step b) with brain-derived neurotrophic factor; and

d) co-culturing the cells of step c) with astrocytes;

wherein said cells are cultured according to steps 1) through d) for at least seven days at each step.

2-4. (Canceled)

5. (Original) The method of claim 1, wherein the stem cells are mammalian stem cells.

6. (Original) The method of claim 1, wherein the stem cells are human stem cells.

7-12. (Canceled)

13. (Previously Presented) A method for inducing cells to differentiate into neuronal cells comprising co-culturing the cells with astrocytes, said cells having gone through the steps of:

a) culturing stem cells with basic fibroblast growth factor;

b) culturing the cells of step a) with fibroblast growth factor 8 and Sonic Hedgehog; and

c) culturing the cells of step b) with brain-derived neurotrophic factor;

wherein said cells are cultured according to steps a) through c) for at least seven days at each step.

EVIDENCE APPENDIX

(1) Schematic diagrams of protocols for producing neuronal cells, astrocytes, and oligodendrocytes, disclosed in Studer and Lee.

(2) Copy of Declaration and *Curriculum Vitae* of Dr. Catherine M. Verfaillie, Publications List, and one literature reference (Snykers et al.) submitted with Appellants' response dated June 18, 2009.

(3) Examiner's Citations and Appellants' Comments.

(4) Wikipedia Entries for Astrocyte and Glial Cell.

RELATED PROCEEDINGS APPENDIX

(None)

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Evidence Appendix (1)

Studer Protocols

DOPAMINERGIC

#	¶	PROTOCOL				
		I	II	III	IV	V
14	15	→	→	→	→ bFGF SHH FGF8 laminin	→
15	15	→	→	→	→ bFGF SHH FGF8 laminin ascorbate	→
16	15	Add any of 15 factors to 14 and 15 above at step III and/or step IV; BDNF is one of these.				
17	31 (F5)	→	→	→	→ fn or laminin bFGF FGF8 SHH	→ AA RA cAMP laminin
18	76	→	→	→	→ bFGF laminin SHH FGF8	→
19	77	Add any of 17 factors to 18 above at step IV and/or V; BDNF is one of these.				
20	85	→	→	→	→ bFGF SHH FGF8 laminin	→
21	87	Add any of five factors to 20 at step V; BDNF is one of these.				

285933

ASTROCYTE

#	¶			PROTOCOL				
		I	II	III	IVA	IVB	V	
28	17	→	→	→	→ laminin SHH FGF8 bFGF	→ bFGF or EGF or PDGF	→	
29	78	I	II	III	IVA	IVB	IVC	V
		→	→	→	→ bFGF	→ bFGF + EGF	→ bFGF + CNTF	→

285986

GABA-ergic

#	¶	PROTOCOL				
		I	II	III	IV	V
27	19	→	→	→	→ laminin bFGF	→ AMP + NT4 or AMP + BDNF

285987

OLIGODENDROCYTE

#	¶		PROTOCOL				
		I	II	III	IVA	IVB	V
25	18, 78	→	→	→	→ laminin bFGF SHH FGF8	→ bFGF EGF	→
26	18, 78	→	→	→	→ laminin bFGF SHH FGF8	→ bFGF CNTF	→

285985

SEROTONERGIC

#	¶	PROTOCOL				
		I	II	III	IV	V
22	16	→	→	→	→ bFGF	→

285982

Lee Protocols

DOPAMINERGIC

#	¶	PROTOCOL				
		I	II	III	IV	V
1	16	→	→	→	→ bFGF	→
2	16	→	→	→	→ EGF	→
3	16	SHH, FGF8 added to 1, 2 above at step IV				
4	17	Can add ascorbate at step V to 1, 2, 3 above				
5	22	→	→	→	→ bFGF laminin	→
6	25 (F4)	→	→	→	→ SHH FGF8	→ AA
7	40	→	→	→	→ bFGF SHH FGF8	→
8	124-125	→	→	→	→ bFGF SHH FGF8	→
9	126	→ SHH FGF8	→ SHH FGF8	→	→	→
10	128	→	→	→	→ bFGF	→ AA
11	200	→	→	→	→ bFGF SHH FGF8 laminin	→

#	¶	PROTOCOL				
		I	II	III	IV	V
12	204	→	→	→	→ bFGF SHH FGF8 laminin	→ laminin
13	205 (F4)	→	→	→	→ bFGF	→ SHH FGF8

#	¶	PROTOCOL				
		I	II	III	IV	V
12	204	→	→	→	→ bFGF SHH FGF8 laminin	→ laminin
13	205 (F4)	→	→	→	→ bFGF	→ SHH FGF8

SEROTONERGIC

#	¶	PROTOCOL				
		I	II	III	IV	V
23	223-226	→	→	→	→ SHH (bFGF)	→
24	223-226	→	→	→	→ SHH FGF8 (bFGF)	→

285983

Evidence Appendix (2)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of

Applicant(s)	:	Catherine M. Verfaillie et al.
Application No.	:	10/561,826
Filed	:	October 17, 2006
Title	:	Neuronal Differentiation of Stem Cells
Examiner	:	Chang Yu Wang
Art Unit	:	1649
Attorney Docket	:	890003-2006.1

DECLARATION UNDER UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Catherine M. Verfaillie, Ph.D., declares and states:

I am Professor of Medicine and Director Stem cell Institute, KULeuven, Leuven I am a co-inventor on the above-captioned patent application.

I am the subject of the attached *Curriculum Vitae* and author of the publications listed on the attachment to the *Curriculum Vitae*. On the information and facts contained in those documents, I submit that I am an expert in the field of Stem cell research. In view of these credentials, I believe that I am qualified to speak on the skill and knowledge of the person of ordinary skill in these fields.

I have read and understand the subject matter of the above-captioned patent application. I have read the first Office Action, dated March 17, 2008, and the second Office Action, dated December 24, 2008. I have read and understand the references cited by the Examiner to support the rejections in the Office Actions. These include U.S. 2003/0211605 to Lee et al. ("Lee") and WO 02/086073 to Studer et al. ("Studer").

It is my opinion, based on the scientific evidence and reasoning below, that the rejections are based on an incorrect assumption about (1) how cells should respond to basic fibroblast growth factor (bFGF), fibroblast growth factor 8 (FGF8), Sonic Hedgehog (SHH), and brain-derived neurotropic factor (BDNF) when these are administered simultaneously as opposed to when these are administered sequentially, and (2) the cell type being acted upon by bFGF, FGF8, SHH, and BDNF in the cited art as opposed to the claimed method.

In the earlier Office Action, on page 6, the Examiner rejects the claims as being obvious over Studer in view of Lee. In the later Office Action, the Examiner provides particular reasons for this rejection. Specifically, the Examiner appears to believe that one would generally expect the same result whether one adds all factors simultaneously or sequentially. Specifically, the Examiner states on page 7 of the Office Action, "...at the end of the final steps, the culture medium contains the identical growth factors as those in Studer's to induce neuronal differentiation." The Examiner then reasons, "...because at the end the culture medium still contains the same growth factors and the same cultured ES cells, ...would be induced to differentiate into neurons." (Emphasis added.) Essentially, the Examiner appears to take the position that sequential exposure should have no significant effect because the factors and the cells exposed to them are the same. I do not agree with this reasoning for the reasons that follow.

The End Product of Sequential Exposure to Factors Cannot be Reasonably Predicted Based on the Results of Simultaneous Exposure to the Same Factors

When one introduces a cocktail of factors, as Studer and Lee have done, all the factors are exposed at once to one discrete cell type as to functional, transcriptional, translational, and morphological characteristics. Thus, the factors are acting at the same time on the same cell. Such exposure produces a certain end product. But, in the claimed methods, there are three phenotypically discrete cell types that are being acted upon: (1) the starting cells, (2) the cells that have been exposed to bFGF but not to FGF8 and SHH, and (3) the cells that have been exposed to bFGF, FGF8 and SHH, but not BDNF. Each mitogen would have a specific effect on the cell to produce a phenotypically discrete cell type. So, if one

exposes a cell to bFGF, FGF8, SHH, and BDNF at the same time, the FGF8/SHH is not acting on a cell with a phenotype created by exposure only to bFGF; and the BDNF is not acting on a cell with a phenotype created by exposure only to bFGF, SHH, and FGF8. Therefore, contrary to what the Examiner asserts, when all the factors are in the medium, they are not acting on the "same cultured ES cells." Because of this, one cannot reasonably predict that the result will be the same as the result obtained by Studer and/or Lee.

Although we have not done an experimental comparison between the Studer and Lee end products and the end products obtained using our own steps a) through c), I will discuss an application of the principle illustrated with a differentiation protocol that we conducted in our laboratory pertaining to differentiation of adult bone marrow stem cells into functional hepatocyte-like cells (Snykers et al., *Toxicological Sciences*, 94:330-341 (2006)). Although the reference is directed to differentiation of stem cells into hepatocytes, the principle applies: sequential exposure to factors can result in quite a different end product than simultaneous exposure ("cocktail").

As reported in this reference, we compared the end product obtained by using a cocktail of factors versus sequential exposure to the factors. I will not go into great detail about this reference as the Examiner is fully qualified to assess the reference. However, I will briefly give an outline of the rationale and the results. It had been previously been shown that bone marrow stem cells could differentiate into hepatocyte-like cells from a simultaneous exposure to a mixture of cytokines and growth factors. To try to improve the end product, the cells were exposed to the same factors in a sequential way. Characterization of the cells over a period of time and after exposure to each factor were characterized in several ways: (1) morphology; (2) mRNA expression of hepatocyte-specific genes; (3) protein expression of hepatocyte-specific genes; and (4) hepatic functionality as assessed by albumin secretion, ureogenesis, glycogen storage, and CYP protein expression activity and inducibility.

Our results showed the following. With respect to morphology, using the sequential procedure, the stem cells acquired morphological features similar to those of primary hepatocytes, particularly polygonal-

shaped and bi-nucleated cells. In contrast, using the previous approach, a heterogenous population of epithelioid cells and other cell types was obtained with no polygonal-shaped cells and only a few bi-nucleated cells. With respect to liver associated genes and proteins, more than 85% of these epithelioid cells expressed these genes and expressed them in a comparable time-dependent manner as observed during *in vivo* liver embryogenesis. In contrast, with the cells exposed to the cocktail, the expression patterns differed from the normal sequence in that HNF1α expression preceded that of albumin. In addition, significantly lower levels of liver-specific markers were expressed. With respect to functional maturation, this occurred with both experimental protocols but to a different extent. Hepatic metabolic functions, including albumin secretion, urea production, etc., were manifested most prominently upon sequential exposure to hepatogenic factors.

This illustrates the principle that the end product of sequential exposure to factors cannot be reasonably predicted based on the results of simultaneous exposure to the same factors.

My understanding is that the rejection of obviousness must be based on motivation to change the Studer/Lee procedure. It is my opinion that one would not have been motivated to alter the procedure of Studer and/or Lee because they would not have reasonably expected to produce the same result. Further, my understanding is that the rejection of obviousness must include a reasonable expectation that the same end product would be obtained. As I understand it, another way of looking at this is that it must be reasonably predictable that the same end product would be produced by simultaneous and sequential exposure to the factors. The Examiner seems to assert that position. But, it is my opinion as an expert in the field, that it was not reasonably predictable that the end product in the prior art would be produced by sequential exposure to the factors.

Summary

In the Studer and Lee method, one discrete cell type (neuronal commitment) is exposed to all three factors at once to create a discrete, functional, morphological, and transcriptional and translational profile. Using

the sequential method, however, the starting cells are first exposed to bFGF, which creates a cell with a specific morphology, function, and transcriptional and translational profile. It is this cell that is acted upon by SHH and FGF8, not the original starting cell as in the "cocktail" method. Then, exposure to FGF8 and SHH produces a cell with a second discrete type of morphology, function, and transcription and translational profile. It is this cell that is acted on by the BDNF, not the original starting cell as in the "cocktail" method. That is what makes the end product not reasonably predictable.

Studer/Lee Do Not Apply the Factors to Multipotent Cells

I also point out that Studer/Lee do not apply bFGF to embryonic stem cells, i.e., to a multipotent stem cell. Studer/Lee apply bFGF to a cell already committed to a neural fate. Studer/Lee form embryoid bodies from embryonic stem cells and grow these embryoid bodies without any differentiation factors, selecting for the cells that have undergone neural commitment. It is those cells that have undergone neural commitment that are exposed to the mitogen(s), SHH, and FGF8. The person of ordinary skill would have expected that neural commitment was needed prior to the application of mitogen(s) SHH and FGF8.

In contrast, in the claimed methods, bFGF is applied directly to multipotent stem cells and not to a neurally committed cell. In fact, it is the bFGF that induces neural commitment in these cells.

For this reason alone, I believe that the claimed method would not motivate one to practice the claimed method.

CONCLUSION

It is my opinion, based on the scientific evidence and reasoning set forth above, that the rejection lacks sufficient scientific basis for finding obviousness.

In my opinion, the person of ordinary skill in this field would not have been motivated to drastically change the approach of Studer and/or Lee from a cocktail approach to a sequential approach and would

not have had a reasonable expectation that the same results would be obtained if they did change the approach.

The Studer/Lee method applied to a neural committed cell, not an embryonic stem cell; therefore, the person of ordinary skill could not have reasonably expected successful application of the Studer/Lee method (i.e., exposure to mitogen(s) FGF8 and SHH) unless embryonic stem cells were committed to a neural fate before the factors were applied.

All statements made herein of my own knowledge are true and all statements made on information believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/1/09
Date

Catherine M. Verfaillie, Ph.D.

11459190.1

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Permanent Resident USA

EDUCATION: M.D.: U. of Leuven Medical School, Leuven, Belgium, 1975-1982

POST-GRADUATE TRAINING:
Internship: 1982-83, AZ St Jan Hospital, Brugge, Belgium
Residency: 1983-85, U. of Leuven, Belgium
Fellow in Hematology: 1985-87, U. of Leuven, Belgium
Post-doctoral Fellow: 1987-89, U. of Minnesota, Minneapolis, MN

PROFESSIONAL APPOINTMENTS:
Instructor, Dep. of Medicine, University of Minnesota, 1989-90,
Assistant Professor of Medicine, University of Minnesota 1991-1995
Associate Professor of Medicine, University of Minnesota 1995-1998
Professor of Medicine, University of Minnesota 1998-
Director Stem Cell Biology Program, University of Minnesota, 1996-1999
Director, Stem Cell Institute, University of Minnesota, 1999-2006
Buitengewoon Hoogleraar, Katholieke Universiteit, Leuven, 2005-
Director, Stamcel Instituut, Katholieke Universiteit, Leuven, 2005-

Member, BME Graduate Program, since 1992
Member, MICaB Graduate Program, since 1992
Member, MD/Ph.D Graduate Program, since 1994
Member Cancer Center, 1994
Member, GCD Graduate Program, since 2000
Member, Clinical Laboratory Science Graduate Program, since 2000
Member, Neuroscience Graduate Program, since 2002
Member Doctoral School, Department of molecular and Cellular Therapy, K.U.Leuven

CERTIFICATION:
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FLEX-1990

HONORS:
M.D., Summa Cum Laude, 1982
Special Fellow, Leukemia Society of America, 1991
Special Fellow, 'Fundacion Internacional Jose Carreras Para La Lucha Contra La Leucemia ',1991
Young Investigator Award, International Society of Exp. Hematology, 1992
Scholar, Leukemia Society of America, 1995
Outstanding Investigator Award, Central Society, 1996
Elected, Member, American Society of Clinical Investigation, 1996
Tulloch Chair in Stem Cell Biology, Genetics and Genomics, 1999
Anderson Chair in Stem Cell Biology, 1999
Elected, Councilor, American Society of Clinical Investigation, 2001
McKnight's Presidential Chair in Stem Cell Biology, 2001
Elected, Member, American Association of Professors, 2003

Vice President, International Society of Experimental Hematology, 2002

President Elect, International Society of Experimental Hematology, 2002
President, International Society of Experimental Hematology, 2004

4th Annual Landazuri Award, University of Navarra, Pamplona, Spain 2002
Damashek Medal, American Society of Hematology, 2002
Honorary Doctorate, Katholieke Universiteit, Belgium, 2003
Distinguished Woman Scholar Award, University Of Minnesota, 2003
2003 Jose Carreras Award, European Society of Hematology, 2003
Forum Engelberg Prize, Lucerne, Switzerland, 2003
Gulden Spoor voor Vlaamse Internationale Uitstraling, Vlaanderen-Europa, 2003
Star Award, Minnesota Hematology Oncology, 2004
Stewart-Niewiarowski Award for Women in Vascular Biology, 2004
Jimenez Diaz Price for Scientific Achievements in Research, 2004
Vlerick Award, 2005
Honorary Member, BeWiSe, 2005
Gabriella Moortgat Prijs, 2006
Bijzonder Hoogleraar, TEFAF Oncology Wisselleerstoel, 2009, Faculty of Health, Medicine, and Life Sciences, Universiteit van Maastricht
Cariplo Professor, University of Pavia, Italy, 2009-2011

NAMED LECTURES:

Presidential Symposium, American Society of Hematology, San Francisco, 2000
Nobel-Forum Lecture series, Karolinska Institute, Stockholm, Sweden, 2000
Linda Laubenstein Memorial Lecture, NYU, New York, 2000
Clement Finch Visiting Professor, University of Washington, Seattle, 2002
4th Annual Landazuri Lecture, University of Navarra, Pamplona, Spain, 2002
Gerhard Smith Memorial Lectureship, City of Hope, CA, 2002
Fiftieth Chalmers J Lyons Memorial Lectureship, AAOMS, Chicago, IL, 2002
Forum Engelberg Award Seminar, Lucerne, Switzerland, 2003
Hohenberg Lecture, university of Pennsylvania, PA, 2003
Kilo Professorship, Washington University, St. Louis, MO, 2003
Evans Lecture, Boston University, Boston, MA, 2003
Stewart-Niewiarowski lecture, Temple University, Boston, MA, 2004
KeKuLe Lecture 2004, Antwerp, Belgium, 2004
NIH Director's Lecture, Washington, DC, 2004
Bendit Lecture, University of Washington, Seattle, WA, 2004
Jimenez Diaz Commemorative Lecture, Madrid, Spain, 2004
Presidential Symposium, ASGT, Minneapolis, MN, 2004
Latta Lecture, University of Nebraska, Omaha, 2005
Presidential Symposium, Am Soc for Investigative Pathology, San Diego, 2005
Moloney Lecture, Brigham & Women's Hospital, Boston MA, 2005
Ada Comstock Inaugural Lecture, Minneapolis, MN, 2005
Brecher Lecture, San Francisco, CA, 2005
Visiting Professor Department of Medicine, Vanderbilt University, 2006
Presidential Symposium, ESTRO, Leipzig, Germany, 2006
Pierre Stryckmans Memorial Lecture, Brussels 2007

PROFESSIONAL ASSOCIATIONS:

American Federation for Clinical Research (AFCR)
American Society of Gene Therapy (ASGT)
American Society of Hematology (ASH)
American Society of Stem Cell and Bone Marrow Transplantation (ASBMT)
International Society for Hematotherapy and Graft Engineering (ISHAGE)
International Society of Experimental Hematology (ISEH)
International Bone Marrow Transplantation Registry (IBMTR)
Autologous Bone Marrow Transplantation Registry (ABMTR)
International Society for Stem Cell Research (ISSCR)

COMMITTEE ASSIGNMENTS (Extramural):

Treasurer, International Society of Experimental Hematology, 1997-2001
Councilor, American Society of Clinical Investigation, 2001-2005
Board of Directors, American Society of Blood and Bone Marrow Transplantation, 2001-2005

Councilor, Society Cell transplantation, 2002-2006
Member, Scientific Committee of the European School of Hematology, 2004-
Chair, Policy Committee, ISSCR, 2002-2006
Member, Scientific Subcommittee on Transfusion Medicine, ASH, 1996-1998
Member, Scientific Subcommittee on Growth Factors, ASH, 1998-2002
Member, Awards Committee, ASH, 2003-2007
Member, Publications Committee, ISEH, 2001-2005
Member, Committee on Hemopoietic Cell and Gene Therapy, ASGT, 98-02
Member, Stem Cell Evaluation Committee, ISHAGE, 1997-2001
Member, Mesenchymal Stem Cell Committee, ISHAGE, 1999-2004
Member, Stem cell Expansion Committee, ISHAGE, 2001-2003

Chair, VA Merit Award Heme Study Section, 2001-2002
Member, NIH study section, Heme I, 2000-2004
Member, VA Merit Award Heme Study Section, 1999-2002
Member, LSA Translational Awards Review Committee, 1999-2004
Member, Telethon Scientific Committee, Italy, 2001-2005
CIRM review committee, 2006-
Review committee, Connecticut Stem Cell Initiative, 2006-
Member CNRS review panel, Belgium, 2008-2012

Ad hoc reviewer, Juvenile Diabetes Research Fund
Ad hoc reviewer, Muscular Dystrophy Association
Ad hoc reviewer, Leukemia Research Fund, Great Britain
Ad hoc reviewer, Welcome Trust, Great Britain
Ad hoc reviewer, Research Council, Canada
Ad hoc reviewer, Nationaal Fonds Wetenschapelijk Onderzoek, Belgium
Ad hoc reviewer, Associazone Italiana per la Ricerca Sul Cancro
Ad hoc reviewer, Israel Science Foundation
Ad hoc reviewer, Dutch Cancer Society
Ad Hoc reviewer, Medical and Health Services Research Division, Ireland.
Ad hoc reviewer, European Commission, FP6
Ad hoc reviewer, European Commission, FP7
Ad hoc reviewer, European Research Council, 2007-

COMMITTEE ASSIGNMENTS (Intramural):
Promotion and Tenure Committee, Department of Medicine 1997-2001
Research Committee, Department of Medicine, since 1997
Steering Committee MD/Ph.D. Program, 1998-2005
Molecular Medicine Planning Committee, 1998-2000
AHC, Functional Genomics Advisory Group, 2000-2004
Consortium on Law and Values in Health, Environment & the Life Sciences, 2001-6

CONSULTANT/ADVISOR
Member International Scientific Advisory Board, UK Government and Wellcome Trust Joint Infrastructure Fund, 1999
Member Advisory Committee, Institute of Hematology, Chinese Academy of Sciences & Peking Union Medical College, 2000-2005
Member, Advisory Committee Tissues of Life Project, Science Museum of Minnesota,2000-2006
Member Scientific Advisory Panel, University of Nebraska Stem Cell Biology Research Center, 1999-2004
Member Scientific Advisory Panel for National Stem Cell Resource, Corriel Institute, Camden, NJ, 2000-2004
Member, Advisory Committee, Mayo Clinic Myeloma PO1, 1998-2004
Member, Stem Cell Advisory Committee, National Research Institute, Taiwan, 2002-
Consultant, Athersys Inc., Cleveland, OH, 2002-
Member, Advisory Board, Center for Transgene Therapy and Gene Therapy, VIB, Leuven, Belgium, 2003
Member Advisory Panel, Stem Cell GAP, NIH, 2003-5
Member, Scientific Advisory Board, Oncostem Therapeutics, Salamanca, Spain, 2004-2007
Member, Scientific Advisory Board, Toronto McLaughlin Centre, 2004-2007
Member, Scientific Advisory Board, Framework 6 Program Beta Cells, 2004
Member, Scientific Advisory Board, DPTE, 2005-
Member, Scientific Advisory Board, Case Western University Stem Cell Institute, 2005-2008
Lid Raad van Advies, EOS, 2005-

Member Advisory Committee, Itinera, 2006-
Advisory Board Regenerative Medicine, the Netherlands, 2007-2009
Co-Chair Research and Quality Assurance Evaluation, Lund University, 2008
Member, Review Committee Science Foundation Ireland REMEDI CSET, 2008
Member, Scientific Advisory Committee, Fondazione Roma, 2008-
Member, Advisory Board, FP7 Infarct Cell Therapy project, PI E Hofer, 2008-
Member International Advisory Board Norway Stem Cell Center, 2008-
Member Advisory Board, EC project "Infarct Cell Therapy", 2009-

ASSOCIATE EDITOR:

Experimental Hematology, 2003-2008
Experimental Hematology, 1998-200
Leukemia, 1997-2002
Hematologia, Citocinas, Immunoterapia Y Terapia Cellular, 1997-2002
Stem Cell Reviews, 2004-2008
PloS-1, 2008-

EDITORIAL BOARD:

Blood, 1995-1999
Experimental Hematology, 1996-1998
Leukemia, 1996-1997
Cytotherapy, 1999-2003
Journal of Biology of Blood and Marrow Transplantation, 2001-2005
Cloning & Stem Cells, 2001-2005
Current Gene Therapy, 2005-
Journal of Engineering and Regenerative Medicine, 2006-
Stem Cells, 2007-2008
Stem Cells International, 2008-

PATENTS:

WO9718298: Ex vivo culture of stem cells
CA2381292: Multipotent adult stem cells and methods for isolation
US2007022482: High-throughput functional analysis of gene expression
WO2006086639: Vascular/lymphatic endothelial cells
WO2006047743: Swine multipotent adult progenitor cells
WO2005045012: Endodermal stem cells in liver and methods for isolation thereof
WO2005003320: Neuronal differentiation of stem cells
WO2008063675: Endodermal progenitor cells
US2002081733: Method to prepare drug-resistant, non-malignant hematopoietic cells
WO2004050859: Homologous recombination in multipotent adult progenitor cells
WO9513088A1: Stroma-derived stem cell growth factors
WO9320184: Method for culturing hematopoietic cells
WO2002040718: Method to identify genes associated with chronic myelogenous leukemia
AU2006304318: Differentiation of non-embryonic stem cells to cells having a pancreatic phenotype
AU2005331534: Use of MAPC or progeny therefrom to populate lymphohematopoietic tissues
US11/808933: High Oct3/4 MAPCs and methods therefor
US 61/022121: Stem cell aggregates and methods for making and using
US08/82108: Optimized methods for differentiation of cells into cells with hepatocyte and hepatocyte progenitor phenotypes, cells produced by the methods, and methods for using the cells
US - GB 0822483.4: Maintenance/expansion of HSCs
US 60/690089 *: HSC Self-Renewal (CIP of US2007022482)

TRAINEES:

High School students		**Current Position**
Evan Cobbs	2002-2003	U of Madison, undergraduate
Nicole Ali	2002-2004	Harvard University, undergraduate
Ricky Jones	2002-2004	CalTech, undergraduate
Sam Bjork	2003-2005	Harvard University, undergraduate

Undergraduate Students		**Current Position**
Venita Chandra	1997-1998	Medical School, U of Chicago

Sarah Aldrich	2000, 2001	Medical School, U of Chicago
Aaron Lisberg	2001, 2002	MD/PhD program,
Zach Kastenberg	2002-2004	Medical School, U. of Minnesota
Eric Rarhman	2002-2005	Graduate School, U of Minnesota
Lee Sandquist	2003-2005	Medical School, U of Minnesota
Juliana Hagenbrock	2003-2006	PhD Student, University of Amsterdam, The Netherlands
April Breyer	2003-2006	Law School, Boston College Law School
Jennifer Gravelle	2003-2005	Medical School, U of Minnesota
Mike Felten	2003-2005	Medical School, U of Minnesota
Thomas Szynski	2003-2005	Medical School, Harvard U

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Sjoban Keel, B.S.	1997-1998	Fellow, Hematology, U. of Washington
Troy Lund, Ph.D.	1998-2002	Pediatric Heme-Onc Fellow, U of Minnesota
David Dyle, B.S.	1998-2000	Fellow, Genetics, U. of Washington
Zubaid Rafique, B.S.	2003-2005	
Tzu-Fei Wang. M.S.	2004-2005	
Eleanor Chen, Ph.D	2004-2006	Pathology Residency, Harvard U

Medical Resident Research		**Current Position**
Jade Anderson, M.D.	1999-2000	Fellow, Hematology, U. of Minnesota
Ken Lee, M.D.	2000-2001	Fellow, Cardiology, UCSF

Medical Resident Advisor		**Current Position**
Sjoban Keel, M.D.	2000-2001	Fellow, Hematology, U. of Washington
David Dyle, M.D.	2000-2004	Fellow, Genetics, U. of Washington

Masters Students		**Current Position**
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Sofia Melikova, B.S.	2000-1002	Scientist, R&D Systems, Minneapolis
Ben Vaessen, B.S.	2000-2002	Scientist, Glaxo-Smith-Kline
Avinash Jayaswal, B.S.	2002-2003	Medical School, UCL, Brussels, Belgium
Qing Cai	2006-2007	PhD Student, K.U.Leuven
Janick Beckers	2006-2007	PhD Student, K.U.Leuven
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Adriaan Campo	2006-2007	PhD Student, U. Antwerpen
Maria Aelberts	2007	PhD Student, U. of Copenhagen
Antonio LoNigro	2007-2008	PhD Student, K.U.Leuven
Simone Calzolari	2007-2008	PhD Student, U. of Barcelona
Kim Van Uytsel	2007-2008	PhD Student, K.U.Leuven
Jasper Wouters	2007-2008	PhD Student, K.U.Leuven
Tine Verryckt	2007-2008	
Olivier Govaere	2007-2008	PhD Student, K.U.Leuven
Lotte Vanbrabant	2007-2008	Technician, K.U.Leuven
Vijay Kumar	2008	PhD Student, U of Toronto
Alessandra Familiari	2008-2009	
Caterina DiPrieto	2008-2009	

Graduate Students:		**Current Position**
Beverly Lundell, MS.	1993-1996	Scientist, Aastrom, Michigan
Eugene Liu, M.D.	1996-2000	Assist Prof of Medicine, U. of Taipei, Taiwan
Scott Dylla, M.S.	1998-2002	Post-doc, Irving Weissman, Stanford U
Fernando Ulloa, B.S	2002-2006	Scientist, Glaxo Smith Kline
Lucas Chase, B.S.	2003-2006	Scientist, Invitrogen
Eric Mendenhall, B.S.	2003-2006	Post-doc, Bernstein lab, MIT
Lepeng Zeng, B.S.	2003-2006	Scientist, Medtronic
Jeff Ross, B.S., M.S.	2003-2006	Scientist, Surmodics
Ben Kidder, B.S.	2003-2007	Postdoc, Serono, Boston
Shannon Buckley, B.S.	2004-2009	
Annelies Crabbe, BS	2006-	
Valerie Roobroeck, BS	2006-	

Kartik Subramanian, BS	2006-	
Yonsil Park, BS	2006-	
Jason Owens, BS	2007-	
Qing Cai, BS, MS	2007-	
Kim Van Uytsel, BS, MS	2008-	
Antonio LoNigro, BS, MS	2008-	
Rojin Abraham MD	2008-	
Elda Mineola, MD	2008-	

MD/PhD students:		**Current Position**
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Robert Schwartz, B.S.	2000-2004	Fellow Gastroenterology, Harvard University, Boston
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Sarah Frommer, B.S.	2002-2006	Medical School, U of Minnesota
Terri Burns, B.S.	2003-2007	Medical School, U. of Minnesota

Postdoctoral *Fellows*

Chunjin Ding, M.D.	1992-1994	Scientist, Ely Lilly, Indianapolis
Huilin Qi, Ph.D.	1998-2003	Scientist, Yale University
Dean Aguiar, Ph.D.	1999-2001	Staff Scientist, Pharmacia
Yuehua Jiang, MS, MD	1999-2002	Assist. Prof., U of Minnesota
Stephanie Salesse, Ph.D.	2000-2005	Assist. Prof, U of Reims, France
Mo Dao, Ph.D.	2001-2004	Post Doc, N Taylor, Montpellier, France
Troy Lund, MD, Ph.D.	2002-2003	Assist. Prof, Department of Pediatrics, U of Minnesota
Yves Heremans, Ph.D.	2002-2005	Instructor VUB, Belgium
Uma Lakshmipathy, Ph.D.	2002-2005	Staff Scientist, Invitrogen, CA
Beatrice Pelacho, Ph.D.	2002-2005	Instructor, U of Navarra, Pamplona, Spain
Aernout Luttun, Ph.D.	2002-2006	Assist. Prof, K.U.Leuven, Belgium
Miguel Barajas, Ph.D.	2004-2006	Assist. Prof., U of Navarra, Pamplona, Spain
Marta Serafini, Ph.D.	2004-2006	Assist. Prof., Fondazione M.Tettamanti M.De Marchi Onlus, Monza, Italy
Rik Snoeckx, Ph.D.	2005-2009	Postdoctoral fellow, J Cools lab, VIB-KULeuven
Kris Van den Boogaert, Ph.D.	2005-	
Carlos Clavel, Ph.D.	2005-2008	Postdoc, Einstein University, USA
Fernando Ulloa, Ph.D.	2006-2008	Scientist, Glaxo Smith Kline
Martine Geeraerts, Ph.D.	2006-	
Pau Sancho-Bru, Ph.D.	2007-2009	
Takeshi Shimizu, Ph.D.	2007-	
Jeroen DeClercq, Ph.D.	2007-	
Bipasha Bose, Ph.D.	2008-2009	
Anujit Kumar, Ph.D.	2008-	
Satish Kumar, Ph.D.	2009-	
Yong Li, Ph.D.	2008-	

Fellows		**Current Position**
Jeffrey S Miller, M.D.	1991-1994	Prof. of Medicine, Director, Translational Research Program, Cancer center, U. of MN
Pankaj Gupta, M.D.	1992-1995	Prof. of Medicine, U. of MN
Randolph Hurley, M.D.	1992-1995	Staff Physician, Health Partners, St Paul, MN
Ravi Bhatia, M.D.	1992-1996	Prof. of Medicine, Director Stem Cell Program, City of Hope
K.Y. Chiang, M.D., Ph.D.	1993-1995	Assist Prof. Pediatrics, Emory University, Atlanta GA
Vivek Roy, M.D.	1994-1996	Assist. Prof. of Medicine, Mayo Clinic, FL
Robert Zhao, M.D., Ph.D	1995-1998	Professor, Professor, Chinese Acad. of Sci. & Peking Union Medical College
Michael Punzel, M.D.	1996-1998	Assoc Prof., U. of Duesseldorf, Germany
Felipe Prosper, M.D.	1995-1997	Prof. of Medicine and Director Stem Cell Program, U. of Navarra, Pamplona, Spain
Michel Delforge, M.D.	1995-1996	Assoc. Prof, KULeuven, Belgium
Juliet Barker, M.D.	1997-1999	Assoc. Prof. of Medicine, Sloan Kettering Institute, NYC
Ian Lewis, M.D., Ph.D.	1997-1999	Assoc. Prof. of Medicine, U. of Adelaide, Australia
Chris Lamming, M.D.	1999-2002	Pediatric Fellow, Baylor College, Houston, TX
Claudio Brunstein, M.D.	1999-2002	Assist Prof. of Medicine, U of Minnesota

Koen Theunissen, M.D.	1999-2001	Hematologist, Virga Jesse Hospital, Hasselt
Balkrishna Jahagirdar, M.D.	2000-2002	Assist Prof. of Medicine, U of Minnesota
Eugene Liu, M.D., Ph.D.	2000-2002	Assist Prof. of Medicine, U. of Taipei, Taiwan
Mojca Jongen, M.D.	2001-2003	Instructor Hematology, U. of Rotterdam, NL
Anskar Leung, M.D., Ph.D.	2001-2003	Assist. Prof, U of Honk-Kong
Masayuki Oki, M.D.	2003-2006	Assist. Prof, Tokai U, Tokio, Japan
Catherine Flynn, M.D.	2004-2007	Consultant Haematologist, St James's Hospital and Coombe Women's Hospital, Dublin
Karen Pauwelyn, M.D.	2005-2009	Fellow, Hepatology, K.U.Leuven
Helene Schoemans, M.D.	2006-2008	Fellow, Hematology, K.U.Leuven
Philip Roelandt, M.D.	2007-	

INVITED LECTURES (Since 2007)

2007

Keynote Speaker, Basel Switzerland
First Connecticut International Stem Cell Symposium, Hartford, CT
Grand Challenge Meeting 4, Lugano, Switzerland
Symposium on tissue reconstruction, UCL, Brussels, Belgium
8th Advanced Summer Course in Cell - Materials Interactions, Instituto de Engenharia Biomédica, Porto, Portugal
MSC2007, Adult Mesenchymal Stem Cells in Regenerative Medicine, Cleveland, OH
The Second UK Mesenchymal Stem Cell Meeting, U of York, Great Britain
International Symposium "Stem cells, Development and Regulation" Amsterdam, The Netherlands
Symposium on Cardiovascular Regenerative Medicine, NIH, Washington DC, USA
Spanish National Congress of Surgery, San Sebastian, Spain
Scottish Stem Cell Network, Glasgow, Scotland
Interhospital Endocrine Rounds, Montreal, Canada
Research Seminar, Buck Institute, CA
Research Seminar, U Liege, Belgium
Research Seminar, VUB, Brussel

2008

Keynote Lecture, EPISTEM conference, Gent, Belgium
Keynote Lecture, VIB, Blankenberge, Belgium
Keynote Lecture, Italian Society for Biotechnology and Medical Engineering, Rieti, Italy
Keynote Lecture, Wadden Symposium on Diabetes, Texel, the Netherlands
Keynote Lecture, Annual Norwegian Stem Cell Network, Oslo Norway
Keynote Lecture, Itera Conference, Maastricht, the Netherlands
Keynote Lecture, Annual science day of the GROW, Maastricht, The Netherlands
EuroSTELLS, Stem Cell Niche Meeting, Barcelona, Spain
Symposium "Pluripotency and differentiation in embryos and stem cells", Pavia, Italy
The Adult Stem/Progenitor Cell Niche, Brussels, Belgium
Nederlandse Vereniging voor Hematologie, Papendal, the Netherlands
Annual Wound Healing Society Meeting, San Diego, USA
International Stem Cell Meeting, Tel Aviv, Israel
EAE/ESH 2nd symposium on MSC, Mandelieu, France
Summer School, Barsinghause, Germany
Nobel Forum Cancer Stem Cell Conference, Stockholm, Sweden
4th International Conference on Regenerative Hepatology, Dusseldorf, Germany
European Society of Gene Therapy, Brugge, Belgium
Mayo Clinic Stem Cell and Regeneration Symposium, Rochester, MN, USA
The 5th Dubai International Conference for Medical Sciences, Dubai
EU-EPC Roundtable, Brussels Belgium

2009

Plenary Lecture, Annual Meeting, French Society of Blood Transfusion, Strasbourg, France
Plenary Lecture, Annual Meeting , ESGT, Hannover, Germany
The Sanquin Spring Seminar, Amsterdam, the Netherlands
Flanders Bio Seminar Tissue Engineering, Brussels
Research Seminar, UCL, Brussels, Belgium
Research Seminar, U Frankfurt, Frankfurt, Germany
Epiplastcarcinoma Marie Curie RTN network meeting, Leuven, Belgium

MEETING ORGANIZER
Yearly Meeting ISEH, 2005, Glasgow, Scotland
Yearly meeting ISEH, 2006, Minneapolis, MN
Keystone meeting, Stem Cells, 2006, Whistler, Canada
Mesenchymal Stem Cells (1), 2006, Mandelieu, France
Mesenchymal Stem Cells (2), 2008, Mandelieu, France
Muscular Dystrophy Symposium Leuven, October 2, 2008
Stem Cells: Biology and Applications; Sponsored by FP6-STROKEMAP and TEFAF, Leuven, 2009

RESEARCH INTERESTS:

1. NORMAL HEMATOPOIESIS:

- Regulation of normal human hematopoietic stem cell proliferation, differentiation and lineage commitment by cytokines and components of the extracellular matrix using *in vitro* as well as *in vivo* xenogeneic transplant models.
- Molecular characterization of hematopoietic stem cells by functional genomics, and zebrafish model of hematopoiesis

2. PLURIPOTENT STEM CELLS (MAPC, ESC, IPS).

- Purification, expansion and characterization of differentiation to mesodermal, ectodermal and endodermal lineages
- Characterization of molecular determinants of pluripotent and multipotent stem cell phenotype, and of de-differentiation and differentiation
- Evaluation of therapeutic potential in congenital disorders or for the treatment of vascular, neurodegenerative disorders, hepatic disorders and diabetes.

CURRENT FUNDING:

- **PO1-CA-65493-06** (PI P McGlave): Biology and Transplantation of Human Stem cells Project Period: 7/1/2000 - 6/30/2005. Project Leader: project 1; Annual Direct Cost: $210,000/year
- **FWO** (PI Verfaillie) Het potentieel van multipotente adulte progenitor cellen in de vervanging van insuline-secreterende ß-cellen in preklinische modellen van type 1 diabetes. Period 1/1/2007-12/31/2009; annual direct cost: €30,000
- **KUL CoE** (PI Verfaillie) Period: 11/1/2005 - 10/31/2009. Annual Direct Cost: €500,000/year
- **Odysseus Fund** (PI Verfaillie) Period: 12/21/2006 - 12/31/2010. Annual Direct Cost: €1,390,000/year
- **FP6-STREP: STROKEMAP** (PI Verfaillie). Multipotent Adult Progenitor Cells to treat Stroke; Period: 10/1/2006 - 9/30/2009. Total Cost: €2,400,000; Total cost Verfaillie €420,000
- **FP6-STREP: CHRYSTAL,** (partner 6: Verfaillie) Cryobanking of stem cells for human clinical application. Period: 1/1/2007 - 12/30/2009. Total cost Verfaillie: €321,000
- **SBO BRAINSTIM** (PI Verfaillie); non-invasive imaging of stem cells in the brain; Period 10/1/2007-9/31/2011; Total Cost: €2,650,000; Total cost Verfaillie €550,000
- **SBO: IMAGINE** (Partner Verfaillie). Generation of improved paramagnetic particles for stem cell labeling and application in tumor therapy. Period: 1/1/2009 - 12/310/2013. Total cost Verfaillie: €250,000

PENDING FUNDING:

- **FP7: BELISTEM** (Partner Verfaillie). Stem cells suitable for liver regeneration. From the bench to the bed side; Period: 1/1/2010 - 12/30/2014. Total cost partner Verfaillie: €1,100,000
- **FP7: STEMPER** (Partner Verfaillie). Stem cells suitable for therapy of peripheral vascular disease. Period: 1/1/2010 - 12/30/2014. Total cost KULeuven: €1,300,000
- **SBO: HEPSTEM** (PI Verfaillie). Generation of mature hepatocytes from human induced pluripotency stem cells Period: 1/1/2010 - 12/310/2014. Total cost €2,500,000
- **NIH GRANT 10121990** (PI Ekker, Co-I Verfaillie). Genomic Analysis of Hematopoietic Stem Cell Niche Formation, Maintenance & Function. Period: 1/7/2009 – 30/6/2014. Yearly Budget Verfaillie: $125,000
- **Dutch Diabetes Fund** (PI P Devos, Co-I Verfaillie). Human fetal and adult progenitor cells as a source for insulin producing cells. 4/1/20o9 - 31/3/2012. Total cost Verfaillie €250,000

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2. Goovaerts J, Verfaillie C. Prenalterol in the treatment of orthostatic hypotension in the Shy-Drager syndrome. Acta Cardiologica 2: 147-155, 1984. (IF: 0.4)
3. Rummens J, Verfaillie C. Capnocytophaga infections: a risk in the immunocompromised host. Acta Clinica Belgica 39: 2-10, 1984. (IF: 0.58)
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9. Verfaillie CM, McGlave P. Leukemia inhibitory factor (LIF)/human interleukin for DA cells (HILDA) supports the growth of multipotential hematopoietic cells in vitro. Blood 77: 263-270, 1991. (IF: 10.9)
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11. Verfaillie CM, Weisdorf D, Haake R, Hostetter P, Ramsay NKC, McGlave P. Candida infections in bone marrow transplant recipients. Bone Marrow Transplant 8:177-184, 1991. (IF: 2.6)
12. Verfaillie CM, McCarthy JB, McGlave PB. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. J Exp Med 174: 693-703, 1991. (IF: 15.6)
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Sequential Exposure to Cytokines Reflecting Embryogenesis: The Key for *in vitro* Differentiation of Adult Bone Marrow Stem Cells into Functional Hepatocyte-like Cells

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Differentiation of adult bone marrow stem cells (BMSC) into hepatocyte-like cells is commonly performed by continuous exposure to a cytokines-cocktail. Here, it is shown that the differentiation efficacy *in vitro* can be considerably enhanced by sequential addition of liver-specific factors (fibroblast growth factor-4, hepatocyte growth factor, insulin-transferrin-sodium selenite, and dexamethasone) in a time-dependent order that closely resembles the secretion pattern during *in vivo* liver embryogenesis. Quantitative RT-PCR analysis and immunocytochemistry showed that, upon sequential exposure to liver-specific factors, different stages of hepatocyte differentiation, as seen during liver embryogenesis, can be mimicked. Indeed, expression of the early hepatocyte markers alpha-fetoprotein and hepatocyte nuclear factor (HNF)3β decreased as differentiation progressed, whereas levels of the late liver-specific markers albumin (ALB), cytokeratin (CK)18, and HNF1α were gradually upregulated. In contrast, cocktail treatment did not significantly alter the expression pattern of the hepatic markers. Moreover, sequentially exposed cells featured highly differentiated hepatic functions, including ALB secretion, glycogen storage, urea production, and inducible cytochrome P450–dependent activity, far more efficiently compared to the cocktail condition. In conclusion, sequential induction of the differentiation process, analogous to *in vivo* liver development, is crucial for *in vitro* differentiation of adult rat BMSC into functional hepatocyte-like cells. This model may not only be applicable for *in vitro* studies of endoderm differentiation but it also provides a "virtually unlimited" source of functional hepatocytes, suitable for preclinical pharmacological research and testing, and cell and organ development.

Key Words: **bone marrow stem cells; hepatocytes; sequential differentiation; liver-specific growth factors; liver embryonic development; *in vitro*.**

INTRODUCTION

Drug development is aimed at identifying pharmacologically active drug candidates with a favorable toxicologic profile. The increasing number of safety criteria, imposed on newly designed molecules, leads nowadays to the urgent need of *in vitro* techniques in the industry, developed according to the principle of Russell and Burch. To date, several hepatocyte-based *in vitro* models are available, however, they are not yet accepted into regulations, as they still require better characterization and optimization to reach the validation stage. Most primary hepatocyte cultures are in fact hampered by progressive occurrence of differentiation (De Smet *et al.*, 2001; LeCluyse *et al.*, 1996; Rogiers and Vercruysse, 1993). An alternative approach would be the use of postnatal progenitor/stem cells.

Indeed, until recently, it was believed that tissue-specific stem cells could only differentiate into cells of the tissue of origin. However, a number of recent studies have suggested that adult stem cells may overcome germ lineage restrictions and express molecular characteristics of cells of different tissue origin, which has been termed "plasticity" (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000; Vourc'h *et al.*, 2004). For example, hematopoietic cells may acquire characteristics of cardiomyocytes, cells of lung, gut, liver, blood vessels, skin, etc. (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000). This apparent plasticity can at least in some instances be explained by cell fusion (Wang *et al.*, 2003). Other studies have described nonhematopoietic stem cells from bone marrow that are capable of differentiating *in vitro* in cells with mesodermal, ectodermal, and endodermal features (Jiang *et al.*, 2002; Reyes *et al.*, 2001; Yoon *et al.*, 2005). The mechanism through which these cells gain multipotency is not totally understood (Verfaillie, 2000). Multipotent adult progenitor cells, for instance, can be induced to express phenotypic and functional characteristics of hepatocytes; however, the degree of differentiation obtained till now is incomplete (Schwartz *et al.*, 2002).

Therefore, in order to develop an *in vitro* model suitable for pharmaco-toxicological purposes, attempts were made here to

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optimize the differentiation efficiency of nonhematopoietic stem cells from bone marrow into functional hepatocytes.

Liver development is accomplished by a sequential array of biological events. Each step of cell growth and differentiation is tightly regulated by cell autonomous mechanisms and extracellular signals, including cytokines and growth factors. More specifically, during the initial phase of murine liver ontogeny (embryonic days [E] 8–9), fibroblast growth factors (FGFs), derived from adjacent cardiac mesoderm, commend the foregut endoderm to form the liver primordium (Duncan, 2000; Jung *et al.*, 1999). During and after the mid-stage of hepatogenesis, surrounding mesenchymal cells secrete hepatocyte growth factor (HGF) and support as such the fetal hepatocytes (Kinoshita and Miyajima, 2002; Zaret, 2002). Around E11, the fetal liver becomes the major site for hematopoiesis. During this stage, hematopoietic stem cells produce oncostatin M that, in the presence of glucocorticoids, not only promotes fetal hepatic cell differentiation and maturation but also suppresses embryonic hematopoiesis. In contrast, oncostatin M alone fails to induce differentiated liver phenotypes, implying that glucocorticoids are essential triggers for hepatic maturation (Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002). In rodents, the final step of hepatic differentiation takes place several days after birth. The lack of terminal differentiation of primary hepatocytes in culture evidences that additional signals, probably generated through the extracellular matrix, are necessary (Kinoshita and Miyajima, 2002).

Here, the liver development was taken as exemplar to establish a culture model that more readily supports robust differentiation of bone marrow stem cells (BMSC) to mature hepatocyte-like cells. We compared two experimental setups: (1) BMSC were treated with a cocktail of liver-specific factors (FGF-4, HGF, insulin-transferrin-sodium selenite [ITS], and dexamethasone [Dex]) as previously described (Schwartz *et al.*, 2002) or (2) innovative in this field, BMSC were exposed to a sequence of these compounds in a manner that closely reflects their temporal expression during *in vivo* hepatogenesis (FGF-4, followed by HGF, followed by a combination of HGF, ITS, and Dex).

MATERIALS AND METHODS

Isolation and culture of undifferentiated rat BMSC. BMSC were isolated from male Fisher rats (4–6 weeks old) and cultured as described by Jiang *et al.* (2002). Labware used for expansion of BMSC included Corning 75 and 150 cm^2 tissue culture flasks, polystyrene (both from VWR, Leuven, Belgium). Cell karyotyping, neuroectodermal, and endothelial differentiation were determined as previously described (Jiang *et al.*, 2002, 2003; Reyes *et al.*, 2001). Rats had access to food and water *ad libitum* and were housed according to guidelines from the Institutional Animal Care and Use Committee of the University of Minnesota.

Hepatocyte differentiation. Rat BMSC from 60 population doublings on were used for differentiation into hepatocyte-like cells. BMSC were plated at 2.1×10^3 cells/cm^2 on 1 mg/ml collagen type I–coated culture plates and dishes (BD Falcon 24-well plate, polystyrene; BD Falcon 35 × 10 mm petri dishes, polystyrene; NUNC F96 microwell plate, black, polystyrene; NUNC F96 microwell plate, clear, polystyrene [all from VWR]) in low-serum expansion medium (Jiang *et al.*, 2002; Reyes *et al.*, 2001). Once cells reached 100% confluence, they were washed with basal medium (Jiang *et al.*, 2003) supplemented with 0.03mM nicotinamide, 0.25mM sodium-pyruvate and 1.623mM glutamine (all from Sigma, Bornem, Belgium). Subsequently, cells were cultured in the presence of liver-specific cytokines and growth factors, added either as a cocktail (basal medium + 10 ng/ml FGF-4, 20 ng/ml HGF [all from R&D Systems, Minneapolis, MN], 1 × ITS and 20 μg/l Dex [all from Sigma]) or sequentially (days 0–3: basal medium + 10 ng/ml FGF-4; days 3–6: basal medium + 20 ng/ml HGF; from day 6 on: basal medium + 20 ng/ml HGF + 1× ITS and 20 μg/l Dex). Differentiation media were changed every 3 days. A schematic presentation of the differentiation procedure is shown in Figure 1.

Quantitative RT-PCR. For PCR analysis, 1 μg RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase and random hexamer primers (Invitrogen, Merelbeke, Belgium). The resulting RT-products were essentially amplified as previously described (Jiang *et al.*, 2003; Schwartz *et al.*, 2002). Three extra steps were included to ensure the purity of the PCR products: 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. The primers used for amplification and the products expected are described in (Jiang *et al.*, 2003; Schwartz *et al.*, 2002). The RNA levels were normalized using 18S and compared with the RNA levels in undifferentiated BMSC (negative control) and freshly isolated primary rat hepatocytes (positive control). As a negative control for the primers, a no template cDNA-PCR reaction was run under the same conditions. The authenticity and size of the PCR products were confirmed by melting curve analysis (using software provided by Perkin Elmer, Lennik, Belgium) and gel electrophoresis.

Immunocytochemistry. Differentiated BMSC were fixed either with ethanol for 10 min at − 20°C (cytoskeletal proteins) or with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 10 min at 4°C, followed by incubation with 100mM glycin to saturate reactive groups (nuclear and cytoplasmic markers). The fixed cells were permeabilized for

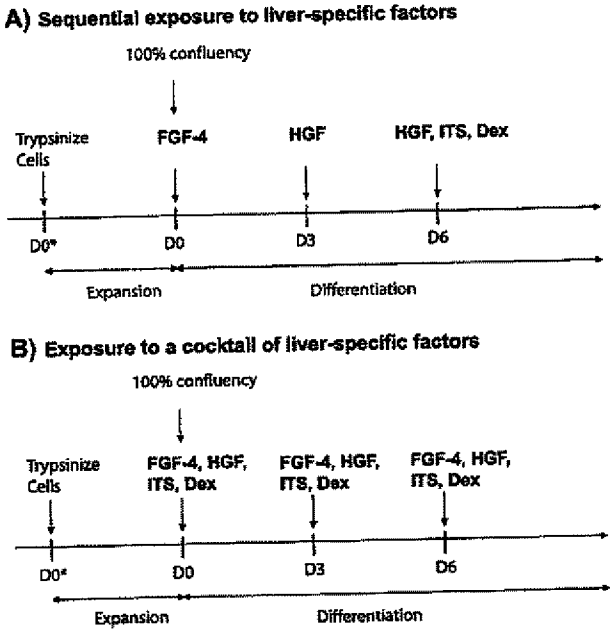

FIG. 1. Schematic presentation of the differentiation protocol. BMSC, at 100% confluency, were exposed either sequentially (A) or simultaneously (B) to liver-specific factors. D0*, day that BMSC were plated at 2.1×10^3 cells/cm^2 on collagen type I in low-serum expansion medium.

15 min with 0.1% Triton in phosphate-buffered saline (Electron Microscopy Sciences) and blocked for 30 min with 1% bovine serum albumin/5% donkey serum block buffer at room temperature. After blocking, cells were incubated overnight at 4°C with primary antibody (fluorochrome-conjugated or non-conjugated) and washed three times with phosphate-buffered saline. In case the primary antibody was not conjugated, cells were incubated for 2 h at room temperature with secondary fluorochrome-conjugated antibody. After incubation, slides were washed again with 0.1% Triton in phosphate-buffered saline and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). As a negative control, cells were incubated with appropriate gamma immunoglobulines (Jackson Immunoresearch, Cambridgeshire, UK) and immunostained under the same conditions. In order to evaluate the localization of cytochrome P450 (CYP) proteins, mitochondria and endoplasmic reticulum were counterstained with the carbocyanine dye $DiOC_6$ (Molecular probes, Invitrogen). Cells were analyzed using fluorescence microscopy with a Zeiss Axiovert scope. To enumerate the number of cells expressing a given marker, all nuclei of positive-stained cells were counted and compared to the total number of cells evaluated. The primary antibodies against alpha-fetoprotein (AFP) (goat), hepatocyte nuclear factor (HNF)3β (goat), and HNF1α (rabbit) were purchased from Santa Cruz, (Heidelberg, Germany). Anti-cytokeratin (CK)18 (mouse, FITC-conjugated) and anti-albumin (ALB) (goat, FITC-conjugated) antibodies were from Sigma and Bethyl Laboratories (Montgomery, TX), respectively. The antibodies against CYP1A1 and CYP2B1/2 (both goat) came from Daiichi pure chemicals, BD Biosciences (Tokyo, Japan). Respective secondary antibodies were purchased from Jackson Immunoresearch.

Albumin ELISA. ALB concentrations, secreted into the culture media, were analyzed by ELISA (Koebe *et al.*, 1994).

Urea assay. The produced urea concentrations were, after 24-h exposure of the cells to 6mM NH_4Cl, colometrically measured in culture media according to the manufacturer's instructions (Quantichrom Urea assay kit, Bioassay Systems, Brussels, Belgium). Fresh culture media supplemented with 6mM NH_4Cl and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Glycogen storage. Intracellular glycogen was analyzed by Periodic-acid-Schiff staining (PAS-kit 395B-1KT, Sigma) according to the manufacturer's instructions. Amyloglucosidase (Sigma)-treated cells and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Alkoxyresorufin-O-dealkylase assay. Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activities were assessed as previously described (Donato *et al.*, 1993) with some minor modifications: in our setup, cells were incubated with 20μM 7-ethoxyresorufin and 18μM 7-pentoxyresorufin (all from Sigma) for 30 min.

To evaluate the inducibility of CYP2B1/2 and CYP1A1/2, respectively, cells were, after 24 days of differentiation, exposed to phenobarbital (PB; final concentration 1mM) and 3-methylcholantrene (MC; final concentration 2μM; all from Sigma). Media, supplemented with either PB or MC, were daily renewed from that time on. Fresh culture media and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Statistics. Results are expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA and Student's *t*-test. The significance level was set at 0.05.

RESULTS

Characterization of the Differentiation Pattern of Rat BMSC into Hepatocyte-like Cells: Sequential versus Cocktail Exposure

Morphological Features

Previously, it has been shown that BMSC could differentiate into hepatocyte-like cells upon simultaneous exposure to a mixture of well-defined cytokines and growth factors (Schwartz *et al.*, 2002). However, using this approach, a rather heterogeneous population of epithelioid cells and other cell types was obtained. Moreover, no polygonal-shaped cells and only few binucleated cells were formed (Fig. 2). In an attempt

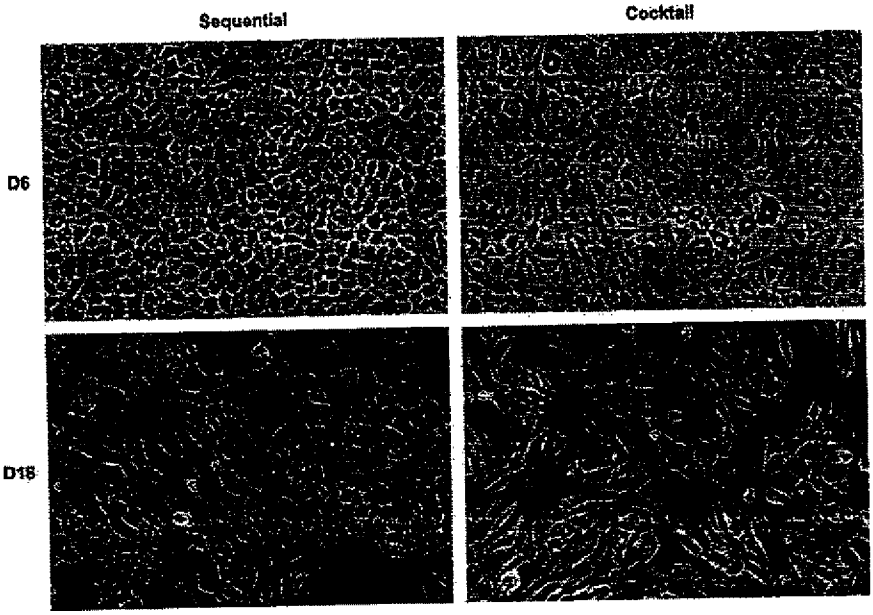

FIG. 2. Light-microscopic analysis of BMSC-derived hepatocyte-like cells upon sequential or simultaneous exposure to liver-specific factors at days 6 and 18. Original magnification of 20 × 10, phase contrast.

to improve the differentiation of nonhaematopoietic stem cells from bone marrow into hepatocyte-like cells, BMSC were exposed to the same well-defined hepatogenic factors, but in a sequential way. More specifically, cytokines and growth factors were added at defined points in time, in a manner that closely resembles the *in vivo* process of embryonic liver development as specified in "Materials and Methods" section (Duncan, 2000; Jung *et al.*, 1999; Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002). In this novel setup, epithelioid cells appeared in culture from day 6 on (Fig. 2). However, at that moment these cells were still surrounded by spindle-shaped cells. After 14 days, less fibroblastic cells were seen and some binucleated cells appeared. After 18 days, most cells exhibited a polygonal shape (Fig. 2).

Characterization at the Molecular Level

In a next set of experiments, we evaluated whether these morphological differences were associated with distinct patterns of differentiation at the molecular level. Therefore, the expressions of early (AFP and HNF3β) and late (ALB, CK18, and HNF1α) liver-specific markers were analyzed at both the mRNA (Fig. 3) and protein levels (Figs. 4 and 5).

mRNA expression. In both sequential and cocktail culture conditions, AFP, HNF3β, ALB, CK18, and HNF1α were expressed in a time-dependent manner during BMSC differentiation. Both the pattern and the level of expression, however, differed considerably between the culture methods. In fact, upon sequential exposure to liver-specific factors, maximal AFP mRNA expression occurred after 6 days (Fig. 3), 4 days later than seen in the cocktail condition, but was 1.2-fold higher than the maximal level observed in cocktail-exposed cells. AFP mRNA expression disappeared completely in both conditions by day 11 of culture. In sequentially treated cells, downregulation of AFP mRNA expression was nicely followed by a second transient, though more pronounced, induction of the early liver-specific marker HNF3β as well as by a steady upregulation of the late hepatic markers ALB, CK18, and HNF1α (Fig. 3). More specifically, HNF3β mRNA expression started at day 2, reached maximal levels at day 10 and decreased rapidly thereafter (Fig. 3). ALB and CK18 mRNA expression, on the other hand, gradually increased from days 4 and 10 of culture, respectively, until maximal levels were reached at day 18 (Fig. 3). In sharp contrast to these observations, changes in HNF3β, ALB, and CK18 mRNA levels were negligible upon simultaneous exposure to all hepatogenic factors ($p < 0.001$; one-way ANOVA). In addition, the mRNA of the late liver-specific marker ALB remained very low in cocktail-exposed BMSC, suggesting an immature hepatic differentiation status. Finally, upon sequential exposure, HNF1α

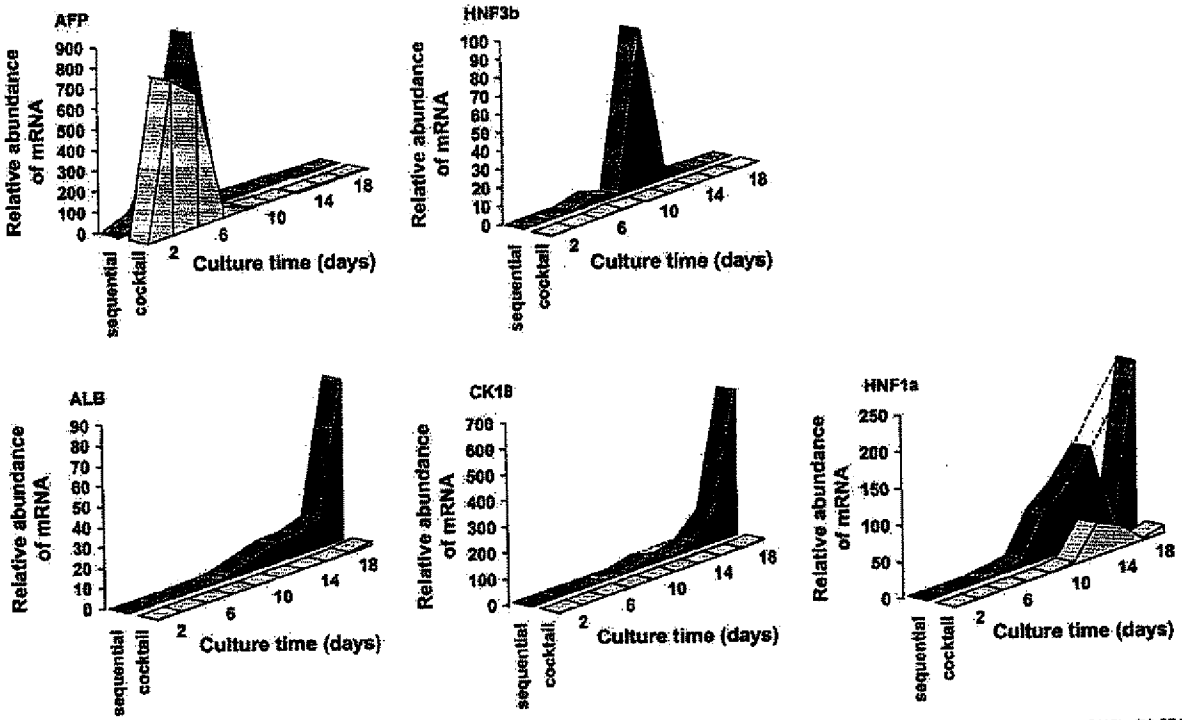

FIG. 3. Analysis of hepatocyte differentiation at the mRNA level (abundance in cultured cells relative to freshly isolated rat hepatocytes [%]). BMSC were either sequentially or simultaneously exposed to liver-specific factors. Values represent means of three independent experiments. The dotted lines represent nontested time points. They are assumptions, based on the existing expression pattern.

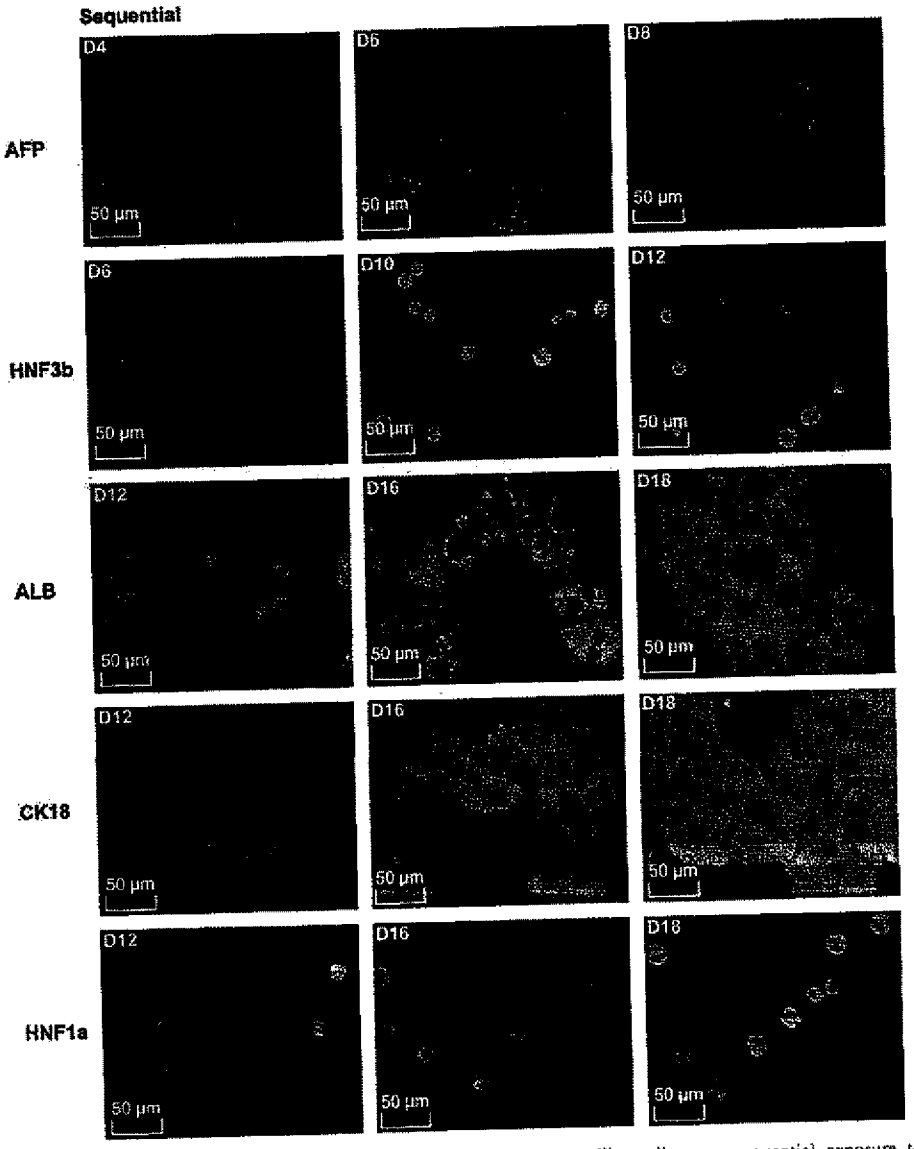

FIG. 4. Characterization at the protein level of BMSC differentiation into hepatocyte-like cells upon sequential exposure to liver-specific factors. Immunocytochemistry was performed for AFP-cy3, HNF3β-cy3, CK18-FITC, HNF1α-cy3, and ALB-FITC. Nuclear counterstaining was assessed using DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for at least five separate experiments.

mRNA expression gradually increased from day 6 on whereas in the cocktail condition, HNF1α mRNA induction was delayed by 4 days and occurred only transiently (Fig. 3). Moreover, maximal levels, obtained at day 12, were about twofold lower than the levels observed in 12-day–old BMSC in the sequential condition. Thus, sequentially exposed BMSC underwent a consecutive array of developmental stages comparable with *in vivo* hepatogenesis while exposure to a cocktail of cytokines and growth factors induced an aberrant expression pattern of differentiation when compared to liver embryogenesis.

Protein expression. In order to support the results obtained at the mRNA level, immunocytochemistry analyses were performed in parallel (Figs. 4 and 5). After 4 days of differentiation, cells expressed AFP, regardless of the experimental setup (Figs. 4 and 5). Concomitantly to the results found at the mRNA level (Fig. 3), AFP expression occurred only transiently in both conditions (Figs. 4 and 5) and was undetectable by day 12 of culture (data not shown). Upon sequential exposure to liver-specific factors, a maximal positive staining of HNF3β (92 ± 8%) was noticed at day 10, leveling off thereafter (Fig. 4). Treatment with all factors

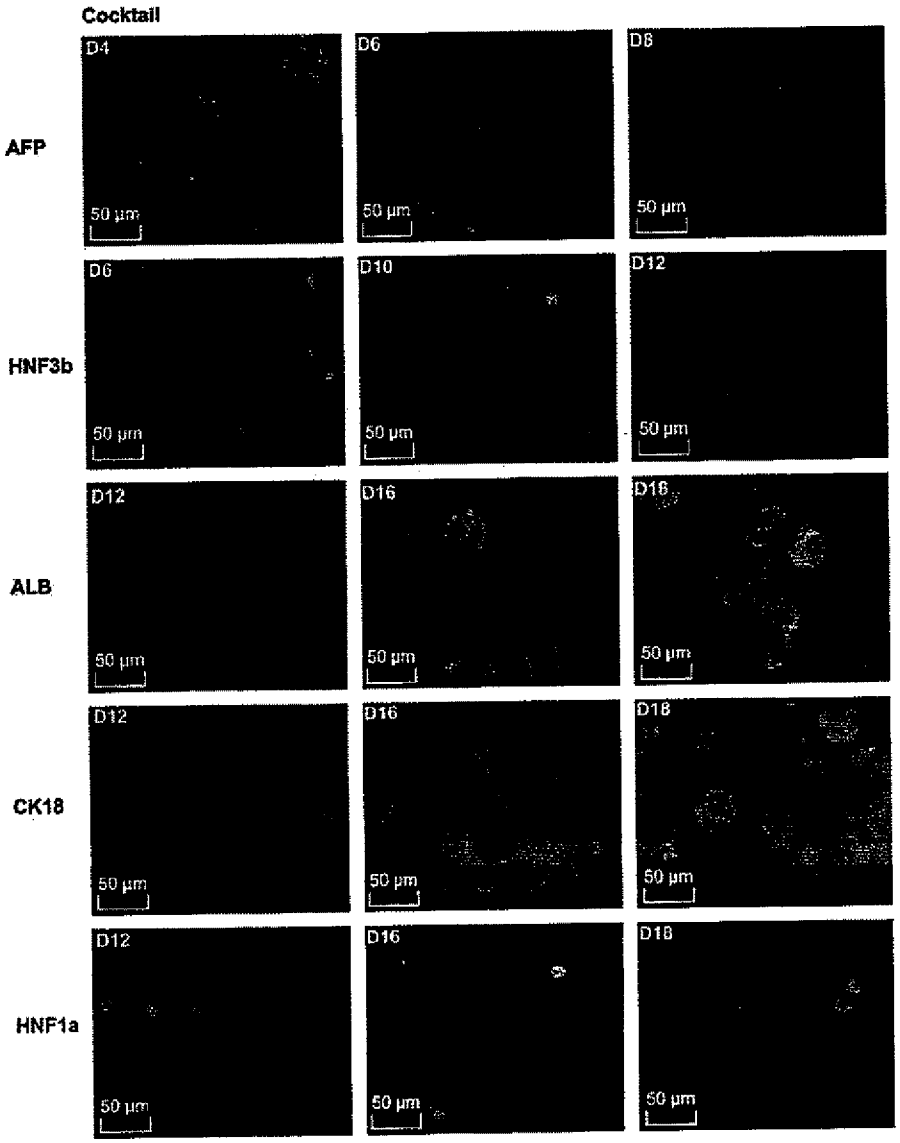

FIG. 5. Characterization at the protein level of BMSC differentiation into hepatocyte-like cells upon simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for AFP-cy3, HNF3β-cy3, CK18-FITC, HNF1α-cy3, and ALB-FITC. Nuclear counterstaining was assessed using DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for at least five separate experiments.

simultaneously, however, revealed no more than 24 ± 7% HNF3β-positive cells throughout the culture period (Fig. 5). As differentiation progressed, extensively increased stainings for ALB, CK18, and HNF1α were detected upon sequential exposure to cytokines and growth factors, in accordance with the results obtained at the RNA level (Figs. 3 and 4). Consequently, after 18 days, 92 ± 2%, 94 ± 3%, and 89 ± 9% of the cells, respectively, stained positive for these markers (Fig. 4), which is in sharp contrast to only 32 ± 4%, 63 ± 5%, and 22 ± 4% of the cocktail-exposed cells, respectively ($p < 0.001$; Student's t-test) (Fig. 4).

In addition, in order to state the immunocytochemistry data with certainty, immunoblotting has been performed in parallel once (data not shown). In line with the previous results obtained at both the mRNA and protein level, sequentially exposed cells expressed liver-specific proteins more abundantly than cells in the cocktail setup. However, since this approach consumes large numbers of cells, i.e., at least 25–50 µg of protein is needed to analyze one liver-specific marker at one point in time, the analysis was not repeated. Alternatively, as measuring CYP activity (the set of EROD/PROD) and their inducibility are widely accepted as final end point to evaluate the suitability of

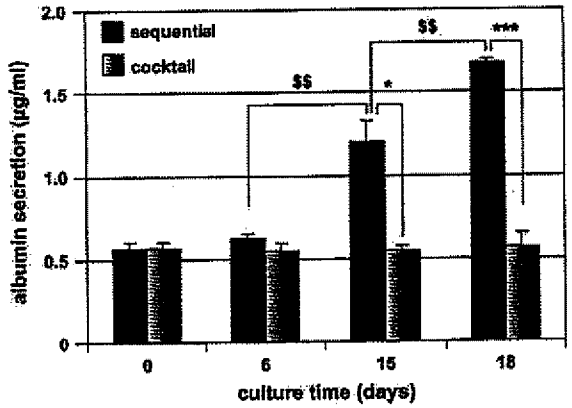

FIG. 6. ALB secretion in sequentially- and cocktail-exposed BMSC. The results shown are representative for five independent experiments, each performed in duplicate. *, ***: ALB-secretion significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ and $p < 0.001$, respectively (Student's t-test). $$: ALB-secretion in sequentially exposed BMSC is significantly upregulated from day 15 on with $p < 0.01$ (Student's t-test).

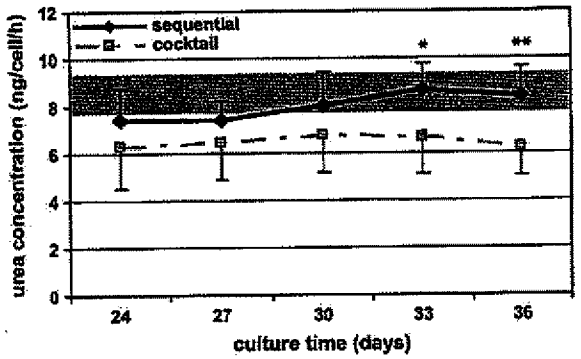

FIG. 7. Urea production in sequentially- and cocktail-exposed BMSC. Gray area represents urea levels, produced by 4 h-cultured adult rat hepatocytes. The graph is representative for four separate experiments, each performed in duplicate. *, **: Urea production significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ and $p < 0.01$, respectively (Student's t-test).

cells as *in vitro* models for pharmaco-toxicological screening of drugs (De Smet *et al.*, 2001; Donato *et al.*, 1993, 2003; LeCluyse *et al.*, 1996; Rogiers and Vercruysse, 1993), we enlarged, in a next set of experiments, the data set on cell functionality in order to increase confidence in our data.

Hepatic Functionality

In order to assess whether these hepatocyte-like cells derived from the bone marrow also acquired typical functional hepatic features, ALB secretion, ammonia metabolism, glycogen storage, expression of CYP proteins in parallel with their activity and inducibility were evaluated.

ALB secretion. Sequentially treated BMSC significantly upregulated the ALB secretion rate from day 15 onward ($p < 0.01$, Student's t-test) (Fig. 6). On the contrary, BMSC exposed to a cocktail of liver-specific factors did not secrete ALB above basal levels, corresponding to 0.55 µg/ml (Fig. 6).

Ureogenesis. Upon sequential exposure to hepatogenic factors, the urea production increased over culture time, reaching adult levels after 30–33 days. In contrast, cocktail-exposed cells synthesized, even at peak production, 24% significant lower urea levels ($p < 0.05$; Student's t-test) (Fig. 7).

Glycogen storage. Furthermore, upon sequential treatment with cytokines and growth factors, glycogen uptake was first seen after 21 days of culture, 6 days earlier than in the cocktail condition. After 30 days of culture, about 86% of the cells stored glycogen, regardless of the culture method (Fig. 8).

CYP protein-expression, activity, and inducibility. In the sequential setup, phase I CYP1A1 and CYP2B1/2 proteins were expressed within and nearby the endoplasmic reticulum and mitochondria (Fig. 9). The level of expression gradually increased as differentiation progressed. After 30 days, 78 ± 1 and 79 ± 3% of the cells stained positive for CYP1A1 and CYP2B1/2, respectively (Fig. 10). In sharp contrast to these observations, cocktail-exposed cells only showed modest CYP expression over culture time (Figs. 9 and 10).

In addition, we investigated whether CYP1A1 and 2B1/2 were functionally active by measuring the respective EROD and PROD activities in both conditions (Figs. 11 and 12).

In line with the results found at the protein level, sequentially exposed cells exhibited markedly higher EROD and PROD activity rates compared to the cocktail model ($p < 0.05$ at days 36 and 39; Student's t-test) (Figs. 11 and 12). Upon sequential exposure to liver-specific factors, a transient fourfold increase in PROD activity was displayed by days 27– 30, approaching the level of 4 h-cultured adult rat hepatocytes, versus only a twofold increase after cocktail treatment (Fig. 11). In addition, in the former setup, EROD activity gradually increased from days 27 to 36 towards levels measured in 4 h-cultured adult rat hepatocytes, whereas CYP1A1/2-dependent activities appeared only transiently in cocktail-exposed cells between days 30 and 33 and declined to almost nondetectable levels on day 36 (Fig. 12).

CYP-inducibility is considered as the most representative metabolic function of the adult hepatic phenotype (Gomez-Lechon *et al.*, 2004; Rogiers and Vercruysse, 1993). Therefore, the responsiveness of both CYP1A1/2 and CYP2B1/2 to their respective prototype inducers MC and PB was analyzed in parallel. PROD activities were induced up to 1.4-fold after 6-day exposure to PB (i.e., on day 30), regardless of the experimental setup (Fig. 11). The inducibility persisted for 6 days in sequentially exposed cells but not in the cocktail condition. A significant CYP1A1/2-dependent response to MC was observed on days 36–39 ($p < 0.001$ and $p < 0.01$ at days 36 and 39, respectively; Student's t-test) in the sequential model. Conversely, MC barely induced EROD activities upon culture with all liver-specific factors simultaneously (Fig. 12).

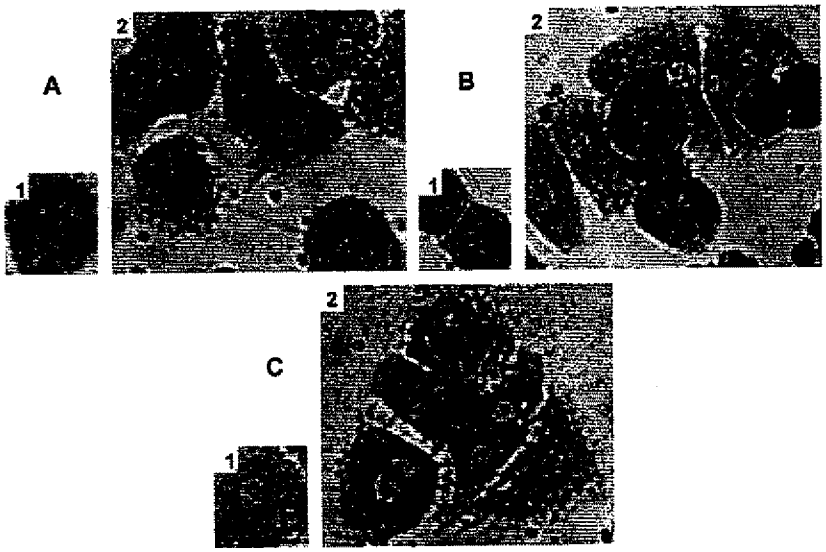

FIG. 8. Glycogen storage in 30-day–old sequentially- (A) and cocktail-exposed BMSC (B) and 4 h-cultured adult rat hepatocytes (C), in the presence (1) and absence (2) of amyloglucosidase, respectively. Glycogen and nuclei are colored magenta and blue, respectively. Original magnification of ×400. Stainings shown are representative for three separate experiments.

DISCUSSION

In recent years, adult-derived stem cells have become a hot topic in the field of molecular, cellular, and clinical biology, as well as in pharmaco-toxicology. Indeed, stem cells have an extensive self-renewing potential and many of them are considered multipotent (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000; Vourc'h *et al.*, 2004). This interest in adult stem cells has in particular been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in preclinical and clinical research (Henningson *et al.*, 2003; McLaren, 2001). The best-characterized stem cell compartment is the bone marrow consisting of two stem cell populations, referred to as the hematopoietic and the mesenchymal

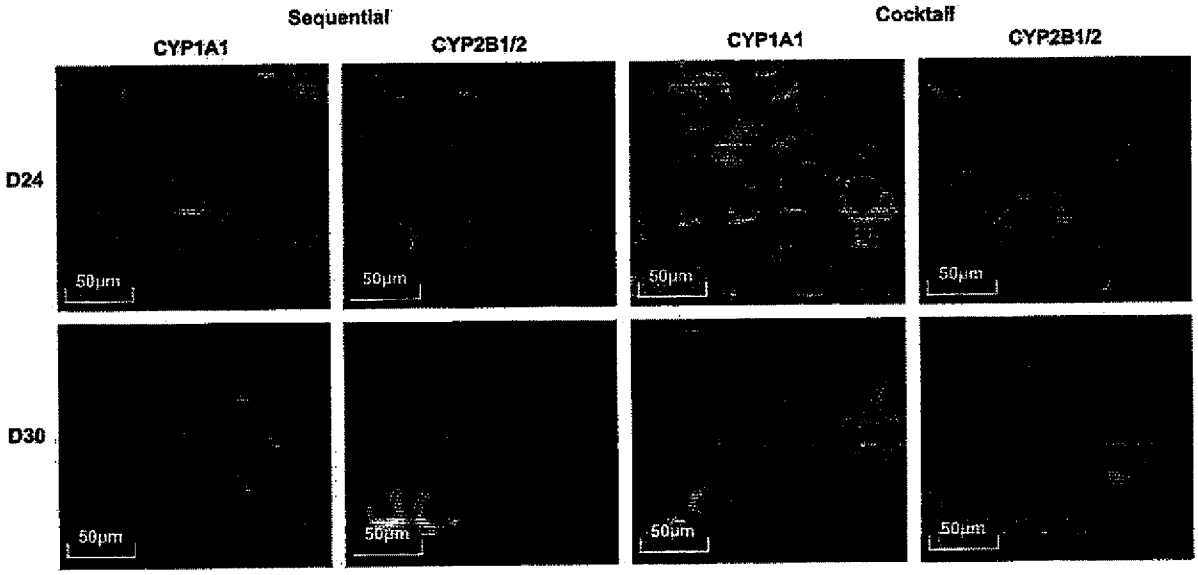

FIG. 9. Detailed view of expression of phase I biotransformation enzymes at days 24 and 30 upon sequential or simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for CYP1A1-cy3 and CYP2B1/2-cy3. Mitochondria and endoplasmic reticulum were counterstained with a green fluorescent carbocyanine. Nuclei were counterstained with DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for three separate experiments.

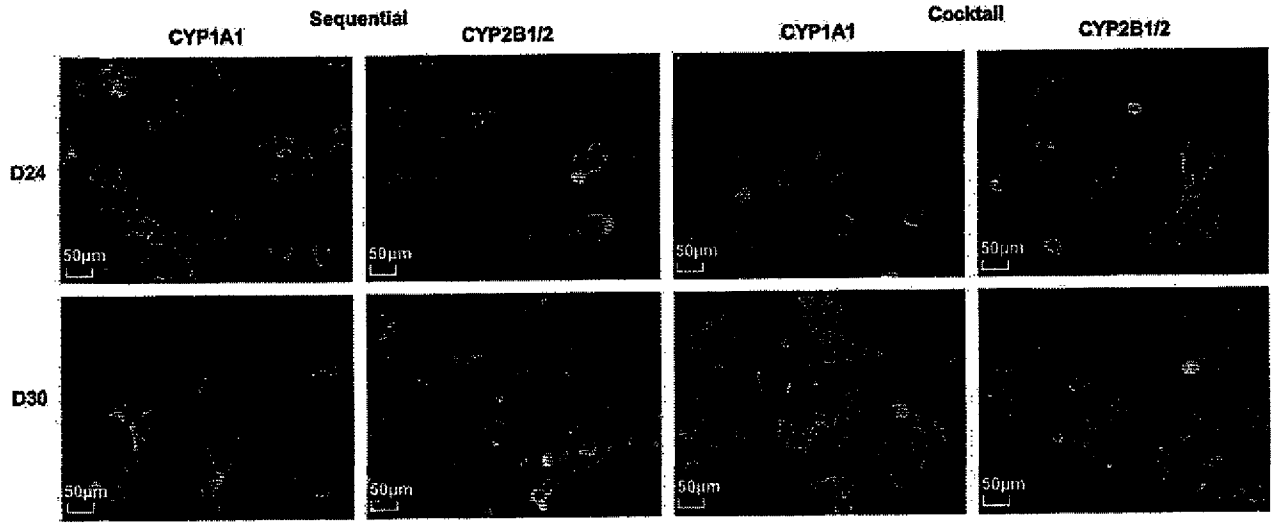

FIG. 10. Overall view of expression of phase I biotransformation enzymes at days 24 and 30 upon sequential or simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for CYP1A1-cy3 and CYP2B1/2-cy3. Mitochondria and endoplasmic reticulum were counterstained with a green fluorescent carbocyanine. Nuclei were counterstained with DAPI. Magnification of ×112. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for three separate experiments.

stem cells (Huttmann *et al.*, 2003). Previously, Schwartz *et al.* (2002) described a population of cells in postnatal rat bone marrow, copurified with mesenchymal stem cells, that were capable of differentiating into cells of endodermal (hepatocytes) origin upon exposure to well-defined hepatogenic factors. These culture conditions yielded, however, a mixture of epithelioid cells and other cell types. Therefore, attempts were made here to improve the hepatic differentiation process through exposure of BMSC to the same liver-specific factors in a sequential time-dependent manner, reflecting their secretion during *in vivo* hepatogenesis (Duncan, 2000; Jung *et al.*, 1999; Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002).

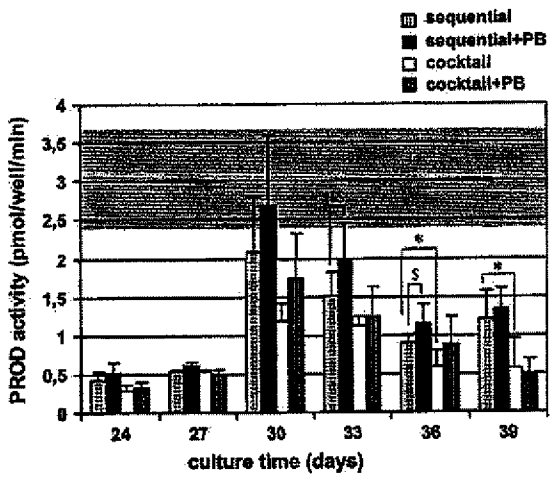

FIG. 11. PROD activities and responsiveness to 1mM PB in differentiated BMSC upon sequential or simultaneous exposure to liver-specific factors. PB was added daily, starting on day 24. Gray area represents PROD activity measured in untreated 4 h-cultured adult rat hepatocytes. The graph is representative for five separate experiments, each, performed in duplicate. *: PROD activity significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ (Student's *t*-test). $, $$: PB significantly induced PROD activity of sequentially-exposed BMSC with $p < 0.05$ and $p < 0.01$, respectively (Student's *t*-test).

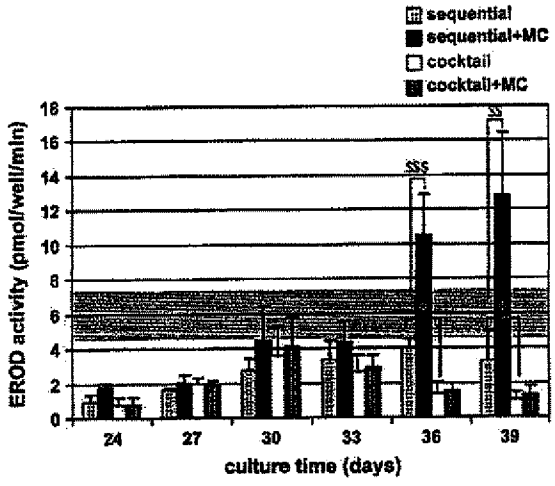

FIG. 12. EROD activities and responsiveness to 2µM MC in differentiated BMSC upon sequential or simultaneous exposure to liver-specific factors. MC was added daily, starting on day 24. Gray area represents EROD activity measured in untreated 4 h-cultured adult rat hepatocytes. The graph is representative for five separate experiments, each performed in duplicate. *: EROD activity significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ (Student's *t*-test). $$, $$$: MC significantly induced EROD activity of sequentially exposed BMSC with $p < 0.01$ and $p < 0.001$, respectively (Student's *t*-test).

Under these culture conditions, BMSC acquired morphological features (polygonal-shaped and binucleated cells) similar to those of primary hepatocytes (Ferrini *et al.*, 1997; Katsura *et al.*, 2002). Furthermore, more than 85% of these epithelioid cells expressed liver-associated genes and proteins (AFP, HNF3β, ALB, CK18, and HNF1α) in a comparable time-dependent manner as observed during *in vivo* liver embryogenesis. Indeed, AFP expression is first detected in embryonic endoderm around E8.5 (Cascio and Zaret, 1991) and precedes ALB and HNF1α expression, detected around E9.5 and E10.5, respectively (Ott *et al.*, 1991; Shiojiri, 1981). This finding implicates that, in this setup, the BMSC differentiation process could serve as a model of early mammalian endoderm differentiation. In contrast, upon exposure to a cytokine/growth factors-cocktail, the expression patterns differed from the normal sequence seen during *in vivo* hepatogenesis as HNF1α expression preceded that of ALB. Indeed, HNF1α is only expressed in fully differentiated cells and not in un- or dedifferentiated cells (Cereghini *et al.*, 1988), as was noticed here upon cocktail treatment. In addition, significantly lower levels of liver-specific markers were expressed. The higher levels of ALB and CK18 expression in the sequential condition are probably due to the higher levels of both the early (HNF3β) and late (HNF1α) transcription factors. It is well documented that liver-enriched transcription factors act cooperatively and synergistically to promote liver-specific gene transcription (Cereghini *et al.*, 1992; Darlington, 1999; Duncan, 2000; Hayashi *et al.*, 1999; Shim *et al.*, 1988). In this regard, it was previously shown that HNF3β positively regulates the expression of HNF4α and HNF1α (Darlington, 1999; Duncan *et al.*, 1998). Furthermore, it is believed that HNF3β serves as the initiator of a cascade of regulatory events resulting in endoderm induction (Ang *et al.*, 1993; Darlington, 1999; Duncan, 2000; Levinson-Dushnik and Benvenisty, 1997). Hence, the minor changes in HNF3β expression levels in the cocktail condition may only result in low levels of ALB and CK18 transcripts and protein.

The initiation and induction of AFP expression is not yet completely understood. It can be assumed that additional factors are involved in its transcriptional activation, as in both culture conditions, only minimal levels of HNF3β were detected at the time of AFP expression. Further research will be needed to fully elucidate the transcriptional hierarchy mediating differentiation of BMSC toward hepatocytes.

The presence of both morphologic and phenotypic features, similar to that of primary hepatocytes, does, however, not fully prove the differentiation of BMSC into mature hepatocytes. Indeed, during the terminal step of liver organogenesis, the liver becomes a functional and metabolic organ, performing an essential role as detoxifying center of the body (Gomez-Lechon *et al.*, 2004; Kinoshita and Miyajima, 2002; Zaret, 2002). Interestingly, functional maturation occurred in both experimental setups, but to a different extent. Hepatic metabolic functions, including ALB secretion, urea production, storage of glycogen, and CYP-activity/inducibility, were manifested most prominently upon sequential exposure to hepatogenic factors. Under these culture conditions, ALB secretion was in fact significantly upregulated to levels comparable to those obtained in both 2- to 7-day–old immobilization and 7-day–old monolayer cultures of primary rat hepatocytes. The latter measurements are performed on a regular basis in our laboratory (Beken *et al.*, 2001; Vanhaecke *et al.*, 2004). In addition, both the urea production and EROD/PROD activities reached levels comparable to 4 h-cultured primary rat hepatocytes. Response to prototype inducers was as expected: pronounced upon exposure to MC and discrete upon PB treatment. The level of induction, however, remained lower in comparison to cultured adult rat hepatocytes. More specifically, EROD activity increased up to fourfold after 15-day exposure to MC in sequentially exposed BMSC versus maximal sevenfold in 2-day treated rat hepatocytes (Donato *et al.*, 1993). Nevertheless, to our best knowledge, this is the first time that EROD (CYP1A1/2) activity/inducibility is demonstrated in hepatocyte-like cells derived from BMSC.

The less mature phenotype of cocktail-exposed cells could possibly be ascribed to altered and lower expression of HNF-type liver-enriched transcription factors in this setup. Experiments using hepatoma cell lines and HNF-null mice have in fact demonstrated the important role of HNFs in the regulation of genes that are involved in biotransformation (*Cyps*) and ammonia metabolism (ornithine-transcarbamylase gene) (Gomez-Lechon *et al.*, 2004; Inoue *et al.*, 2002; Rodriguez-Antona *et al.*, 2002). Similar to the results reported here, inducible CYP2B1/2-activity was also found by Schwartz *et al.* (2002) after exposure to a cocktail of the same cytokines and growth factors, although at an earlier time in culture. Some variation in time-specific gene and protein expression could probably be attributated to intraspecies differences and subtle changes in the differentiation procedure (i.e., type of culture plate coating, serum, etc.).

In summary, during the first 18 days of the hepatic differentiation process of BMSC, cells, and sequentially exposed BMSC in specific, underwent a sequential array of developmental stages, characterized by the down- and upregulation of early and late liver-specific markers, respectively. As differentiation progressed, i.e., from day 18 onward, expression of mature hepatic markers persisted at steady levels (data not shown) and cells gradually underwent functional hepatic maturation. In specific, sequentially treated BMSC accomplished hepatic functions at levels comparable to those of primary rat hepatocytes, cultured for 4 h to 2 days. Our results thus clearly show that a more pronounced and homogeneous differentiation of BMSC into functional hepatocyte-like cells can be obtained by sequentially directing the differentiation process analogous to liver embryogenesis. Moreover, differentiation appears to occur via steps commonly defined for *in vivo* endodermal lineage specification and subsequent hepatocyte differentiation and maturation. Further investigations, in order

to elucidate the molecular mechanisms underlying the changes described herein, are underway.

This model opens new perspectives: it may not only be applicable to study endoderm differentiation *in vitro* but it also offers the possibility to purify and culture multipotent stem cells from nonembryonic origin as an unlimited cell source for pharmaco-toxicological research and testing, and cell and organ development. It might even open a road to trigger cell fate and "trans"-differentiate uncommitted cells from different tissues towards endodermal lineages.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

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Evidence Appendix (3)

Office Action Dated March 17, 2008

Lee

Abstract; [116]; [129]; [0016]-[0021]; [0040]-[0043]; [0045]-[0048]; [0058]; [0071]-[0093]; [147]-[148]; Example 1-Example 7. "[Lee] teaches a method of inducing stem cells to differentiate into neuronal cells comprising culturing embryonic stem cells in the present of bFGF, FGF8, SHH, and co-culturing cells with astrocytes as recited in instant claims 1 and 13." Page 8 of the Office Action.

Comment: None of this text discloses any procedure as recited in claims 1 and 13, even as they were originally presented, for the reasons discussed in Appellants' Brief.

"[Lee] teaches differentiation of cultured embryonic stem cells into neurons...and the differentiation of stem cells encompasses 3% astrocytes, which meets the limitation of 'comprising co-culture astrocytes' [sic] as recited in instant claims 1 and 13." Page 8 of the Office Action.

Comment: No cite to the reference is given. Appellants are unable to find any disclosure where a differentiation protocol encompasses 3% astrocytes wherein, according to the protocol, these astrocytes are co-cultured with neurons, (i.e., no disclosure of protocols wherein neurons are the end product), also producing 3% astrocytes, and no disclosure that the protocol for generating astrocytes also generates neurons.

[111]. "Although [Lee] does not explicitly teach at least seven days for each step as recited in instant claim 4, the complete culture procedures of [Lee] take more than one month...which meets the limitation of the instant claim 4." Page 8 of the Office Action.

Comment: Paragraph 111 does not even address the time that the entire procedure takes. The paragraph only discusses the first stage wherein the ES cells are first cultured.

[125]-[127]. "[Lee] also teaches differentiation of stem cells into dopaminergic, serotonergic, GABA-ergic neurons and combinations thereof..." Page 9 of the Office Action.

Comment: This text is directed to increasing the efficiency of the generation of dopaminergic neurons by adding factors SHH and FGF8 to control dopaminergic and serotonergic cell fates.

[70]-[71]. "[Lee] also teaches BDNF to promote survival and function of neurons and glial cells." Page 9 of the Office Action.

Comment: This section is directed only to a list of neurologic agents which is a general exposition of what these agents are. Paragraph 71 defines the term "neurologic agent" and lists around 20 of them without pointing to any particular one for any particular function. BDNF is one among them and is not singled out in any way. Paragraph 70 also generally defines the term "neurologic agent" and indicates that it refers to any substance that promotes the function or survival of neurons. It says that, as an example, a preferred neurologic agent (unspecified what that preference is) can promote nerve or glial cell growth, promote survival of functioning cells, augment the activity of functioning cells, enhance the synthesis of neurotransmitter substances, augment the activity of naturally occurring nerve growth promoting factors, act as a nerve growth promoting factor, prevent degeneration of neurons, induce re-growth of dendrite and axon, have more than one of these properties or the like.

[177]. "[Lee] also teaches ...cells transfected with BDNF gene to promote survival and function of neurons." Page 9 of the Office Action.

Comment: This paragraph does not relate to the protocols at all. It is found in a section on gene therapy, indicating that the cultured cells may be transfected with a desired neurologically- relevant polypeptide. BDNF is an example of several genes that are listed. The implication of the Examiner is that the section teaches introducing BDNF into the protocol to promote survival and function of neurons by way of cells transfected with this gene, but, as one can see from the text itself, this is not the case.

Studer

Page 4, line 12 – Page 6, line 20; Page 24, Example 2 – Page 30. "[Studer] teaches a method of inducing stem cells to differentiate into neuronal cells comprising culturing embryonic stem cells in the presence of bFGF,

FGF8, SHH, BDNF, and co-culturing the cells with astrocytes as recited in instant claims 1 and 13." Page 7 of the Office Action.

Comment: For all the reasons given in Appellants' Brief, none of the cited text recites the procedure as claimed.

Pages 25 and 26, Paragraphs 75-78. "[Studer] teaches differentiation of cultured embryonic stem cells into neurons and astrocytes which meet the limitation of 'comprising co-culture astrocytes' [sic]as recited in instant claims 1 and 13." Page 7 of the Office Action.

Comment: Page 25 is part of a section (Example 2) regarding the generation of mid-brain dopaminergic neurons. Paragraphs 75 and 77 have no disclosure that relates to astrocytes. Paragraph 76 indicates that stage V is induced by withdrawal of the mitogen bFGF with subsequent differentiation of ES-derived CNS precursors into differentiated neuronal and glial progeny. It is not at all clear that there is any co-culture of neuronal cells with astrocytes, even if one interprets glial progeny as encompassing astrocytes. Appellants have explained their rationale for this procedure in the Brief. See page 14. Paragraph 78 is not related to co-culturing neurons and astrocytes. It is directed to modifying the protocol to generate astrocytes and other cell types of potential interest, such as GABA-ergic neurons and oligodendrocytes. Accordingly, the protocol is designed to produce astrocytes, not to produce neurons and astrocytes.

Pages 27-29. "In addition, although [Studer] does not explicitly teach at least seven days for each step as recited in instant claim 4, each step in each stage of the culture condition of [Studer] requires 6-9 days and the whole culture procedure takes more than one month...which is within the limitation of instant claim 4." Page 8 of the Office Action.

Comment: This section simply discusses what takes place in each of the stages. According to this section, the factors are only added at stage IV, so in the month to which the Examiner refers, at least three of those five stages do not include exposure of the cells to the four claimed factors. A review of all the permutations in the

schematics for producing dopaminergic neurons in Studer (i.e., Appellants' Evidence Appendix), shows that the greatest theoretical time frame during which all these factors is involved is 14 days.

Office Action Dated October 29, 2009

Studer

Page 5, Paragraph 16-17; Page 26, Paragraph 78. "[Studer] teaches that in order to generate astrocytes before adding SHH and FGF8 into the culture medium, the ES cells are proliferated and cultured in a culture medium in the presence of bFGF." Page 8 of the Office Action.

Comment: Paragraph 16 only generally refers to a method for generating a serotonergic neuron and indicates that, in doing this, the mitogen used is bFGF. Paragraph 17 refers to the protocol that is specific for generating an astrocyte. Here, which is consistent with the Appellants' schematics, the mitogen, SHH, and FGF8 are added together in stage IV and a sequential stage IV is performed where the cell produced in stage IV is then again incubated with a mitogen, which can be bFGF, EGF, and PDGF. Accordingly, bFGF is either added simultaneously with or subsequent to the SHH and FGF8, which is the opposite of what the Examiner has asserted. Paragraph 78 is directed to modifying the differentiation protocol to generate cells other than dopaminergic neurons (i.e., astrocytes) by re-plating the stage IV cells as is stated above; oligodendrocytes, by re-plating the stage IV cells and then proliferating them with bFGF, EGF, or PDGF; and GABA-ergic neurons, where SHH and FGF8 are deliberately not included.

Page 26, Paragraph 78; Page 29, Paragraph 87. "[Studer] also teaches that expanded ES cells from stage IV are induced to neuronal differentiation in the culture medium in the presence of BDNF and the absence of SHH and FGF8..." Page 8 of the Office Action.

Comment: The implication is that Studer teaches steps b) and c) of claim 1. This is correct as far as it goes, but the citations of the Examiner (paragraph 78) are directed to making GABA-ergic neurons where, during the entire protocol, there is no SHH or FGF8. These are explicitly excluded. Accordingly, this would not suggest the

claimed protocol at all. Paragraph 87 does not even discuss SHH and FGF8. It is just directed to stage V (the differentiation stage at which the mitogen is withdrawn) and simply points out that several factors, BDNF among them, can promote dopaminergic differentiation and survival and, therefore, could be added at stage V.

Office Action Dated June 22, 2010

Lee

[125]; [128]; Example 5. "Although the claimed method alters the way of adding growth factors...at the end of the steps the result of neuronal differentiation from stem cells including dopaminergic, serotonergic, and GABA-ergic neurons, is expected at taught by Studer and Lee." Page 4 of the Office Action.

Comment: None of this text indicates that neuronal differentiation would be expected by making the claimed modification. Paragraph 125 simply discusses adding FGF8 and SHH along with the mitogen in stage IV to control dopaminergic and serotonergic cell fate (increase ratio of dopaminergic to serotonergic neurons). Paragraph 128 is simply directed to the differentiation stage (i.e., stage V) by withdrawal of at least neurologic agent. Accordingly, this section does not address the claimed modification or show what would be expected if one were to make such a modification. Example 5, interestingly, teaches away from the claimed procedure as discussed in Appellants' Brief. Example 5 is directed to the generation of dopaminergic neurons. It is indicated that differentiation was induced by withdrawing the mitogen bFGF, that adding SHH and FGF8 during stage IV resulted in an increased number of dopaminergic neurons, and that adding SHH and FGF8 at stage V, instead of stage IV, i.e., not with bFGF, but in the stage after it, "proved ineffective."

Evidence Appendix (4)

Astrocyte

From Wikipedia, the free encyclopedia

For the cell in the gastrointestinal tract, see Interstitial cell of Cajal.

Astrocytes (etymology: astron gk. star, cyte gk. cell), also known collectively as **astroglia**, are characteristic star-shaped glial cells in the brain and spinal cord. They perform many functions, including biochemical support of endothelial cells that form the blood–brain barrier, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord following traumatic injuries.

Neuron: Astrocyte

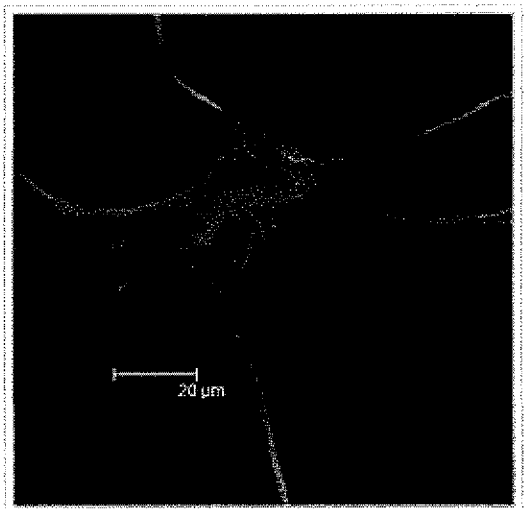
Astrocytes can be visualized in culture because they express glial fibrillary acidic protein.

NeuroLex ID *sao1394521419 (http://www.neurolex.org/wiki/sao1394521419)*

Dorlands/Elsevier *12165688 (http://www.mercksource.com/pp/us/cns/cns_hl_dorlands_split.jsp?pg=/ppdocs/us/common/dorlands/dorland/a_68.htm)*

Research since the mid-1990s has shown that astrocytes propagate intercellular Ca^{2+} waves over long distances in response to stimulation, and, similar to neurons, release transmitters (called gliotransmitters) in a Ca^{2+}-dependent manner. Data suggest that astrocytes also signal to neurons through Ca^{2+}-dependent release of glutamate.[1] Such discoveries have turned astrocyte research into a rapidly growing field of neuroscience.

Contents



Isolated Astrocyte shown with confocal microscopy. Image: MacLean and Ivey

Description

Astrocytes are a sub-type of glial cells in the central nervous system. They are also known as *astrocytic glial cells*. Star-shaped, their many processes envelope synapses made by neurons. Astrocytes are classically identified using histological analysis; many of these cells express the intermediate filament glial fibrillary acidic protein (GFAP). Three forms of astrocytes exist in the CNS, *fibrous*, *protoplasmic*, and *radial*. The fibrous glia are usually located within white matter, have relatively few organelles, and exhibit long unbranched cellular processes. This type often has "vascular feet" that physically connect the cells to the outside of capillary wall when they are in close proximity to them. The protoplasmic glia are found in grey matter tissue, possess a larger quantity of organelles, and exhibit short and highly branched cellular processes. The radial glia are disposed in a plane perpendicular to axis of ventricles. One of their processes about the pia mater, while the other is deeply buried in gray matter. Radial glia are mostly present during development, playing a role in neuron migration. Mueller cells of retina and Bergmann glia cells of cerebellar cortex represent an exception, being present still during adulthood. When in proximity to the pia mater, all three forms of astrocytes send out process to form the pia-glial membrane.

Previously in medical science, the neuronal network was considered the only important one, and astrocytes were looked upon as *gap fillers*. More recently, the function of astrocytes has been reconsidered,[2] and are now thought to play a number of active roles in the brain, including the secretion or absorption of neural transmitters and maintenance of the blood–brain barrier.[3] Following on this idea the concept of a "tripartite synapse" has been proposed, referring to the tight relationship occurring at synapses among a presynaptic element, a postsynaptic element and a glial element.[4]

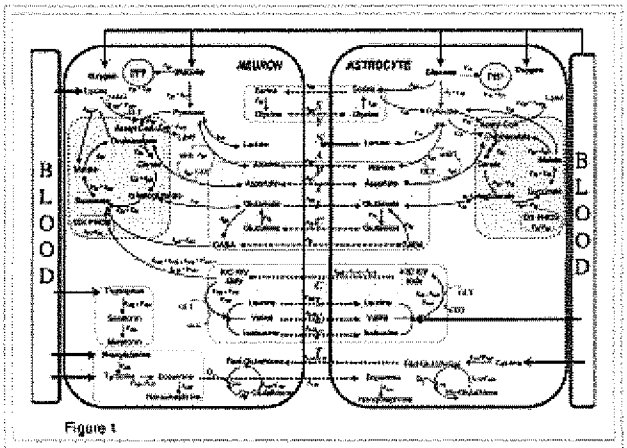
Astrocytes (red) among neurons in the living cerebral cortex

Functions

- **Structural**: They are involved in the physical structuring of the brain. Astrocytes get their name because they are "star-shaped". They are the most abundant glial cells in the brain that are closely associated with neuronal synapses. They regulate the transmission of electrical impulses within the brain.
- **Metabolic support**: They provide neurons with nutrients such as lactate.
- **Blood–brain barrier**: The astrocyte end-feet encircling endothelial cells were thought to aid in the maintenance of the blood–brain barrier, but recent research indicates that they do not play a substantial role; instead, it is the tight junctions and basal lamina of the cerebral endothelial cells that play the most substantial role in maintaining the barrier.[5] However, it has recently been

shown that astrocyte activity is linked to blood flow in the brain, and that this is what is actually being measured in fMRI.[6]

- **Transmitter uptake and release**: Astrocytes express plasma membrane transporters such as glutamate transporters for several neurotransmitters, including glutamate, ATP, and GABA. More recently, astrocytes were shown to release glutamate or ATP in a vesicular, Ca^{2+}-dependent manner.[7] (This has been disputed for hippocampal astrocytes.)[8]
- **Regulation of ion concentration in the extracellular space**: Astrocytes express potassium channels at a high density. When neurons are active, they release potassium, increasing the local extracellular concentration. Because astrocytes are highly permeable to potassium, they rapidly clear the excess accumulation in the extracellular space. If this function is interfered with, the extracellular concentration of potassium will rise, leading to neuronal depolarization by the Goldman equation. Abnormal accumulation of extracellular potassium is well known to result in epileptic neuronal activity.[*citation needed*]
- **Modulation of synaptic transmission**: In the supraoptic nucleus of the hypothalamus, rapid changes in astrocyte morphology have been shown to affect heterosynaptic transmission between neurons.[9] In the hippocampus, astrocytes suppress synaptic transmission by releasing ATP, which is hydrolyzed by ectonucliotidases to yield adenosine. Adenosine acts on neuronal adenosine receptors to inhibit synaptic transmission, thereby increasing the dynamic range available for LTP.[10]
- **Vasomodulation**: Astrocytes may serve as intermediaries in neuronal regulation of blood flow.[11]
- **Promotion of the myelinating activity of oligodendrocytes**: Electrical activity in neurons causes them to release ATP, which serves as an important stimulus for myelin to form. However, the ATP does not act directly on oligodendrocytes. Instead, it causes astrocytes to secrete cytokine leukemia inhibitory factor (LIF), a regulatory protein that promotes the myelinating activity of oligodendrocytes. This suggest that astrocytes have an executive-coordinating role in the brain.[12]
- **Nervous system repair**: Upon injury to nerve cells within the central nervous system, astrocytes fill up the space to form a glial scar, repairing the area and replacing the CNS cells that cannot regenerate.[*citation needed*]
- **Long-term potentiation**: Scientists are arguing back and forth on if astrocytes integrate learning and memory in the hippocampus. We know that glial cells are included in neuronal synapses, but many of the LTP studies are preformed on slices, so that is where scientists are disagreeing on whether or not astrocytes have a direct role of modulating synaptic plasticity.



Metabolic interactions between astrocytes and neurons. From a computational study by Çakır et al., 2007.

Recent studies

A recent study was done in November of 2010 and was published March 2011. It was done by a team of scientists from the University of Rochester and University of Colorado School of Medicine led by Professor Chris Proschel. They did an experiment to attempt to repair trauma to the Central Nervous System of an adult rat by replacing the glial cells. When the glial cells were injected into the injury of the adult rat's spinal cord, astrocytes were generated by exposing human glial precursor cells to bone morphogenetic protein (Bone morphogenetic protein is important because it is considered to create

tissue architecture throughout the body). So, with the bone protein and human glial cells combined, they promoted significant recovery of conscious foot placement, axonal growth, and obvious increases in neuronal survival in the spinal cord laminae. On the other hand, human glial precursor cells and astrocytes generated from these cells by being in contact with ciliary neurotrophic factors, failed to promote neuronal survival and support of axonal growth at the spot of the injury 34.

One study done at Shanghai, they had two types of hippocampal neuronal cultures: In one culture, the neuron was grown from a layer of astrocytes and the other culture was not in contact with any astrocytes, but they were instead fed a Glial Conditioned Medium (GCM) which in most cases in rats, this inhibits the rapid growth of cultured astrocytes in their brain. In their results they were able to see that astrocytes had a direct role in LTP with the mixed culture (which is the culture that was grown from a layer of astrocytes) but not in GCM cultures 36.

Recent studies have shown that astrocytes play an important function in the regulation of neural stem cells. Research from the Schepens Eye Research Institute at Harvard shows the human brain to abound in neural stem cells, which are kept in a dormant state by chemical signals (ephrin-A2 and ephrin-A3) from the astrocytes. The astrocytes are able to activate the stem cells to transform into working neurons by dampening the release of ephrin-A2 and ephrin-A3.[*citation needed*]

Furthermore, studies are underway to determine whether astroglia play an instrumental role in depression, based on the link between diabetes and depression. Altered CNS glucose metabolism is seen in both these conditions, and the astroglial cells are the only cells with insulin receptors in the brain.

Calcium waves

Astrocytes are linked by gap junctions, creating an electrically coupled (functional) syncytium.[13]

An increase in intracellular calcium concentration can propagate outwards through this functional syncytium. Mechanisms of calcium wave propagation include diffusion of calcium ions and IP3 through gap junctions and extracellular ATP signalling.[14] Calcium elevations are the primary known axis of activation in astrocytes, and are necessary and sufficient for some types of astrocytic glutamate release.[15]

Classification

There are several different ways to classify astrocytes.

Lineage and antigenic phenotype

These have been established by classic work by Raff et al. in early 1980s on Rat optic nerves.

- Type 1: Antigenically Ran2$^+$, GFAP$^+$, FGFR3$^+$, A2B5$^-$, thus resembling the "type 1 astrocyte" of the postnatal day 7 rat optic nerve. These can arise from the tripotential glial restricted precursor cells (GRP), but not from the bipotential O2A/OPC (oligodendrocyte, type 2 astrocyte precursor, also called *Oligodendrocyte progenitor cell*) cells.
- Type 2: Antigenically A2B5$^+$, GFAP$^+$, FGFR3$^-$, Ran 2$^-$. These cells can develop **in vitro** from the either tripotential GRP (probably via O2A stage) or from bipotential O2A cells (which some people think may in turn have been derived from the GRP) **or** in vivo when the these progenitor cells are **transplanted** into lesion sites (but *probably not in normal development, at least not in*

the rat optic nerve). Type-2 astrocytes are the major astrocytic component in postnatal optic nerve cultures that are generated by O2A cells grown in the presence of fetal calf serum but are not thought to exist in vivo (Fulton et al., 1992).

Anatomical classification

- Protoplasmic: found in grey matter and have many branching processes whose end-feet envelop synapses. Some protoplasmic astrocytes are generated by multipotent subventricular zone progenitor cells.[16][17]
- Gömöri-positive astrocytes. These are a subset of protoplasmic astrocytes that contain numerous cytoplasmic inclusions, or granules, that stain positively with Gömöri's chrome-alum hematoxylin stain. It is now known that these granules are formed from the remnants of degenerating mitochondria engulfed within lysosomes,[18] Some type of oxidative stress appears to be responsible for the mitochondrial damage within these specialized astrocytes. Gömöri-positive astrocytes are much more abundant within the arcuate nucleus of the hypothalamus and in the hippocampus than in other brain regions. They may have a role in regulating the response of the hypothalamus to glucose.[19][20]
- Fibrous: found in white matter and have long thin unbranched processes whose end-feet envelop nodes of Ranvier.[21] Some fibrous astrocytes are generated by radial glia.[22][23][24][25][26]

Transporter/receptor classification

- GluT type: express glutamate transporters (EAAT1/*SLC1A3 (http://www.genenames.org/data/hgnc_data.php?match=SLC1A3)* and EAAT2/*SLC1A2 (http://www.genenames.org/data/hgnc_data.php?match=SLC1A2)*) and respond to synaptic release of glutamate by transporter currents
- GluR type: express glutamate receptors (mostly mGluR and AMPA type) and respond to synaptic release of glutamate by channel-mediated currents and IP3-dependent Ca2+ transients

Bergmann glia

Bergmann glia, a type of glia[27][28] also known as radial epithelial cells (as named by Camillo Golgi) or Golgi epithelial cells (GCEs; not to be mixed up with Golgi cells), are astrocytes in the cerebellum that have their cell bodies in the Purkinje cell layer and processes that extend into the molecular layer, terminating with bulbous endfeet at the pial surface. Bergmann glia express high densities of glutamate transporters that limit diffusion of the neurotransmitter glutamate during its release from synaptic terminals. Besides their role in early development of the cerebellum, Bergmann glia are also required for the pruning or addition of synapses.[*citation needed*]

Pathology

SLC1A3 expression highlights Bergmann glia in the brain of a mouse at 7th postnatal day, sagittal section.

Astrocytomas are primary intracranial tumors derived from astrocytes cells of the brain. It is also possible that glial progenitors or neural stem cells give rise to astrocytomas.

Astrocytomas are brain tumors that develop from astrocytes. They may occur in many parts of the brain and sometimes in the spinal cord. They can occur at any age but they primarily occur in males. Astrocytomas are divided into two categories: Low grade (I and II) and High Grade (III and IV). Low grade tumors are more common in children and high grade tumors are more common in adults[32].

Pilocytic Astrocytoma are Grade I tumors. They are considered benign and slow growing tumors. Pilocytic Astrocytomas frequently have cystic portions filled with fluid and a nodule, which is the solid portion. Most are located in the cerebellum. Therefore, most symptoms are related to balance or coordination difficulties[32]. They also occur more frequently in children and teens[33].

Grade II Tumors grow relatively slow but invade surrounding healthy tissue. Usually considered benign but can grow into malignant tumors. Other names that are used are Fibrillary or Protoplasmic astrocytomas. They are prevalent in younger people who are often present with seizures[33].

Anaplastic astrocytoma is classified as grade III and are malignant tumors. They grow more rapidly than lower grade tumors and tend to invade nearby healthy tissue. Anaplastic astrocytomas recur more frequently than lower grade tumors because of their tendency to spread into surrounding tissue makes them difficulty to completely remove surgically[32].

Glioblastoma Multiforme is also a malignant tumor and classified as a grade IV. Glioblastomas can contain more than one cell type (i.e., astrocytes, oligondroctyes). Also, while one cell type may die off in response to a particular treatment, the other cell types may continue to multiply. Gliobastomas are the most invasive type of glial tumors. Grows rapidly and spreads to nearby tissue. Approximately 50% of astrocytomas are glioblastomas and are very difficult to treat[32].

Tripartite synapse

Within the dorsal horn of the spinal cord, activated astrocytes have the ability to respond to almost all neurotransmitters (Haydon, 2001) and, upon activation, release a multitude of neuroactive molecules such as glutamate, ATP, nitric oxide (NO), prostaglandins (PG), and D-serine, which in turn influences neuronal excitability. The close association between astrocytes and presynaptic and postsynaptic terminals as well as their ability to integrate synaptic activity and release neuromodulators has been termed the "tripartite synapse" (Halassa et al., 2006). Synaptic modulation by astrocytes takes place because of this 3-part association.

Astrocytes in chronic pain sensitization

Under normal conditions, pain conduction begins with some noxious signal followed by an action potential carried by nociceptive (pain sensing) afferent neurons, which elicit excitatory postsynaptic potentials (EPSP) in the dorsal horn of the spinal cord. That message is then relayed to the cerebral cortex, where we translate those EPSPs into "pain." Since the discovery of astrocytic influence, our understanding of the conduction of pain has been dramatically complicated. Pain processing is no longer seen as a repetitive relay of signals from body to brain, but as a complex system that can be up- and

down-regulated by a number of different factors. One factor at the forefront of recent research is in the pain-potentiating synapse located in the dorsal horn of the spinal cord and the role of astrocytes in encapsulating these synapses. Garrison and co-workers (Garrison, 1991) were the first to suggest association when they found a correlation between astrocyte hypertrophy in the dorsal horn of the spinal cord and hypersensitivity to pain after peripheral nerve injury, typically considered an indicator of glial activation after injury. Astrocytes detect neuronal activity and can release chemical transmitters, which in turn control synaptic activity (Volters and Meldolesi, 2005; Haydon, 2001; Fellin, et al., 2006). In the past, hyperalgesia was thought to be modulated by the release of substance P and excitatory amino acids (EAA), such as glutamate, from the presynaptic afferent nerve terminals in the spinal cord dorsal horn. Subsequent activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid), NMDA (N -methyl-D-aspartate) and kainate subtypes of ionotropic glutamate receptors follows. It is the activation of these receptors that potentiates the pain signal up the spinal cord. This idea, although true, is an oversimplification of pain transduction. A litany of other neurotransmitter and neuromodulators, such as calcitonin gene-related peptide (CGRP), adenosine triphosphate (ATP), brain-derived neurotrophic factor (BDNF), somatostatin, vasoactive intestinal peptide (VIP), galanin, and vasopressin are all synthesized and released in response to noxious stimuli. In addition to each of these regulatory factors, several other interactions between pain-transmitting neurons and other neurons in the dorsal horn have added impact on pain pathways.

Two states of persistent pain

After persistent peripheral tissue damage there is a release of several factors from the injured tissue as well as in the spinal dorsal horn. These factors increase the responsiveness of the dorsal horn pain-projection neurons to ensuing stimuli, termed "spinal sensitization," thus amplifying the pain impulse to the brain. Release of glutamate, substance P, and calcitonin gene-related peptide (CGRP) mediates NMDAR activation (originally silent because it is plugged by Mg2+), thus aiding in depolarization of the postsynaptic pain-transmitting neurons (PTN). In addition, activation of IP3 signaling and MAPKs (mitogen-activated protein kinases) such as ERK and JNK, bring about an increase in the synthesis of inflammatory factors that alter glutamate transporter function. ERK also further activates AMPARs and NMDARs in neurons. Nociception is further sensitized by the association of ATP and substance P with their respective receptors, $[[P_2X_3]]$, and neurokinin 1 receptor (NK1R), as well as activation of metabotropic glutamate receptors and release of BDNF. Persistent presence of glutamate in the synapse eventually results in dysregulation of GLT1 and GLAST, crucial transporters of glutamate into astrocytes. Ongoing excitation can also induce ERK and JNK activation, resulting in release of several inflammatory factors.

As noxious pain is sustained, spinal sensitization creates transcriptional changes in the neurons of the dorsal horn that lead to altered function for extended periods. Mobilization of Ca2+ from internal stores results from persistent synaptic activity and leads to the release of glutamate, ATP, tumor necrosis factor -α (TNF-α), interleukin 1β (IL-1β), IL-6, nitric oxide (NO), and prostaglandin E2 (PGE2). Activated astrocytes are also a source of matrix metalloproteinase 2 (MMP2), which induces pro-IL-1β cleavage and sustains astrocyte activation. In this chronic signaling pathway, p38 is activated as a result of IL-1β signaling, and there is a presence of chemokines that trigger their receptors to become active. In response to nerve damage, heat shock proteins (HSP) are released and can bind to their respective TLRs, leading to further activation.

See also

- Bergmann gliosis

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External links

- Cell Centered Database - Astrocyte (http://ccdb.ucsd.edu/sand/main?stype=lite&keyword=protoplasmic%20astrocyte&Submit=Go&event=display&start=1)
- UIUC Histology Subject *57 (https://histo.life.illinois.edu/histo/atlas/oimages.php?oid=57)*
- "Astrocytes" (http://www.sfn.org/index.cfm?pagename=brainBriefings_astrocytes) at Society for Neuroscience
- The Department of Neuroscience at Wikiversity
- NIF Search - Astrocyte (http://www.neuinfo.org/nif/nifgwt.html?query=%22Astrocyte%22) via the Neuroscience Information Framework

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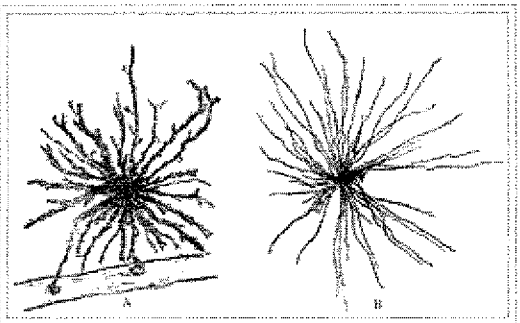
Categories: Nervous system | Glial cells | Human cells

Glial cell

From Wikipedia, the free encyclopedia

Glial cells, sometimes called **neuroglia** or simply **glia** (Greek γλία, γλοία "glue"; pronounced in English either /gli:ə/ or /glaɪə/), are non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for the brain's neurons. In the human brain, there is roughly one glia for every neuron with a ratio of about two neurons for every three glia in the cerebral gray matter.[1]



Neuroglia of the brain shown by Golgi's method.

As the Greek name implies, glia are commonly known as the glue of the nervous system; however, this is not fully accurate. The four main functions of glial cells are to surround neurons and hold them in place, to supply nutrients and oxygen to neurons, to insulate one neuron from another, and to destroy pathogens and remove dead neurons. For over a century, it was believed that they did not play any role in neurotransmission. That idea is now discredited[2]; they do modulate neurotransmission, although the mechanisms are not yet well understood.[2][3][4]

Astrocytes can be identified in culture because, unlike other mature glia, they express glial fibrillary acidic protein.

Contents

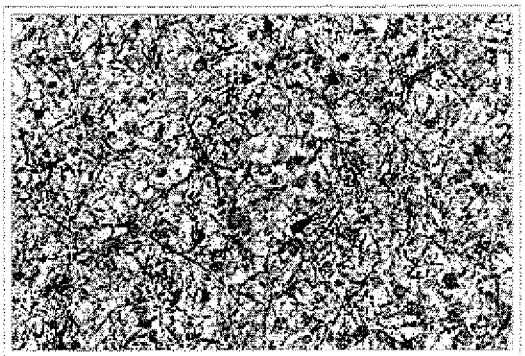
Glial cells in a rat brain stained with an antibody against GFAP.

Functions

Some glial cells function primarily as the physical support for neurons. Others regulate the internal environment of the brain, especially the fluid surrounding neurons and their synapses, and nutrify neurons. During early embryogenesis, glial cells direct the migration of neurons and produce molecules that modify the growth of axons and dendrites. Recent research indicates that glial cells of the hippocampus and cerebellum

participate in synaptic transmission, regulate the clearance of neurotransmitters from the synaptic cleft, release factors such as ATP, which modulate presynaptic function, and even release neurotransmitters themselves.

Glial cells are known to be capable of mitosis. By contrast, scientific understanding of whether neurons are permanently post-mitotic,[5] or capable of mitosis, [6] is still developing. In the past, glia had been considered to lack certain features of neurons. For example, glial cells were not believed to have chemical synapses or to release neurotransmitters. They were considered to be the passive bystanders of neural transmission. However, recent studies have shown this to be untrue.[7]



Neoplastic glial cells stained with an antibody against GFAP (brown). Brain biopsy.

For example, astrocytes are crucial in clearance of neurotransmitter from within the synaptic cleft, which provides distinction between arrival of action potentials and prevents toxic build-up of certain neurotransmitters such as glutamate (excitotoxicity). It is also thought that glia play a role in many neurological diseases, including Alzheimer's disease. Furthermore, at least in vitro, astrocytes can release neurotransmitter glutamate in response to certain stimulation. Another unique type of glial cell, the oligodendrocyte precursor cells or OPCs, have very well-defined and functional synapses from at least two major groups of neurons.[*citation needed*] The only notable differences between neurons and glial cells are neurons' possession of axons and dendrites, and capacity to generate action potentials.

Glia ought not to be regarded as 'glue' in the nervous system as the name implies; rather, they are more of a partner to neurons.[8] They are also crucial in the development of the nervous system and in processes such as synaptic plasticity and synaptogenesis. Glia have a role in the regulation of repair of neurons after injury. In the CNS, glia suppress repair. Glial cells known as astrocytes enlarge and proliferate to form a scar and produce inhibitory molecules that inhibit regrowth of a damaged or severed axon. In the PNS, glial cells known as Schwann cells promote repair. After axonal injury, Schwann cells regress to an earlier developmental state to encourage regrowth of the axon. This difference between PNS and CNS raises hopes for the regeneration of nervous tissue in the CNS. For example a spinal cord may be able to be repaired following injury or severance.

Types

Microglia

For more details on this topic, see Microglia.

Microglia are like specialized macrophages capable of phagocytosis that protect neurons of the central nervous system. They are derived from hematopoietic precursors rather than ectodermal tissue; they are commonly categorized as such because of their supportive role to neurons.

These cells comprise approximately 15% of the total cells of the central nervous system.[*citation needed*] They are found in all regions of the brain and spinal cord. Microglial cells are small relative to macroglial cells, with changing shapes and oblong nuclei. They are mobile within the brain and multiply when the brain is damaged. In the healthy central nervous system, microglia processes constantly sample all aspects of their environment (neurons, macroglia and blood vessels).

Macroglia

Location	Name	Description
CNS	Astrocytes	The most abundant type of macroglial cell, *astrocytes* (also called *astroglia*) have numerous projections that anchor neurons to their blood supply. They regulate the external chemical environment of neurons by removing excess ions, notably potassium, and recycling neurotransmitters released during synaptic transmission. The current theory suggests that astrocytes may be the predominant "building blocks" of the blood-brain barrier. Astrocytes may regulate vasoconstriction and vasodilation by producing substances such as arachidonic acid, whose metabolites are vasoactive. Astrocytes signal each other using calcium. The gap junctions (also known as electrical synapses) between astrocytes allow the messenger molecule IP3 to diffuse from one astrocyte to another. IP3 activates calcium channels on cellular organelles, releasing calcium into the cytoplasm. This calcium may stimulate the production of more IP3. The net effect is a calcium wave that propagates from cell to cell. Extracellular release of ATP, and consequent activation of purinergic receptors on other astrocytes, may also mediate calcium waves in some cases. In general, there are two types of astrocytes, protoplasmic and fibrous, similar in function but distinct in morphology and distribution. Protoplasmic astrocytes have short, thick, highly branched processes and are typically found in gray matter. Fibrous astrocytes have long, thin, less branched processes and are more commonly found in white matter. It has recently been shown that astrocyte activity is linked to blood flow in the brain, and that this is what is actually being measured in fMRI.[9]
CNS	Oligodendrocytes	*Oligodendrocytes* are cells that coat axons in the central nervous system (CNS) with their cell membrane forming a specialized membrane differentiation called myelin, producing the so-called myelin sheath. The myelin sheath provides insulation to the axon that allows electrical signals to propagate more efficiently.[10]
CNS	Ependymal cells	*Ependymal cells*, also named *ependymocytes*, line the cavities of the CNS and make up the walls of the ventricles. These cells create and secrete cerebrospinal fluid(CSF) and beat their cilia to help circulate that CSF and make up the Blood-CSF barrier. They are also thought to act as neural stem cells.[11]

CNS	Radial glia	*Radial glia cells* arise from neuroepithelial cells after the onset of neurogenesis. Their differentiation abilities are more restricted than those of neuroepithelial cells. In the developing nervous system, radial glia function both as neuronal progenitors and as a scaffold upon which newborn neurons migrate. In the mature brain, the cerebellum and retina retain characteristic radial glial cells. In the cerebellum, these are Bergmann glia, which regulate synaptic plasticity. In the retina, the radial Müller cell is the principal glial cell, and participates in a bidirectional communication with neurons.[12]
PNS	Schwann cells	Similar in function to oligodendrocytes, *Schwann cells* provide myelination to axons in the peripheral nervous system (PNS). They also have phagocytotic activity and clear cellular debris that allows for regrowth of PNS neurons.[13]
PNS	Satellite cells	*Satellite glial cells* are small cells that surround neurons in sensory, sympathetic and parasympathetic ganglia.[14] These cells help regulate the external chemical environment. Like astrocytes, they are interconnected by gap junctions and respond to ATP by elevating intracellular concentration of calcium ions. They are highly sensitive to injury and inflammation, and appear to contribute to pathological states, such as chronic pain.[15]
PNS	Enteric glial cells	Are found in the intrinsic ganglia of the digestive system.They are thought to have many roles in the enteric system, some related to homeostasis and muscular digestive processes.[16]

Capacity to divide

Glia retain the ability to undergo cell division in adulthood, whereas most neurons cannot. The view is based on the general deficiency of the mature nervous system in replacing neurons after an injury, such as a stroke or trauma, while very often there is a profound proliferation of glia, or gliosis near or at the site of damage. However, detailed studies found no evidence that 'mature' glia, such as astrocytes or oligodendrocytes, retain the ability of mitosis. Only the resident oligodendrocyte precursor cells seem to keep this ability after the nervous system matures. On the other hand, there are a few regions in the mature nervous system, such as the dentate gyrus of the hippocampus and the subventricular zone, where generation of new neurons can be observed.[*citation needed*]

Embryonic development

Most glia are derived from ectodermal tissue of the developing embryo, in particular the neural tube and crest. The exception is microglia, which are derived from hemopoietic stem cells. In the adult, microglia are largely a self-renewing population and are distinct from macrophages and monocytes, which infiltrate the injured and diseased CNS.

In the central nervous system, glia develop from the ventricular zone of the neural tube. These glia include the oligodendrocytes, ependymal cells, and astrocytes. In the peripheral nervous system, glia derive from the neural crest. These PNS glia include Schwann cells in nerves and satellite glial cells in ganglia.

History

Glia were discovered in 1846 by the pathologist Rudolf Virchow in his search for a 'connective tissue' in the brain. (see as reference: http://physrev.physiology.org/content/81/2/871.long)

Numbers

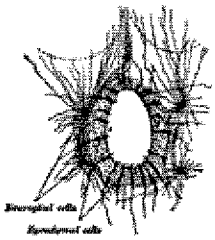
The human brain contains roughly equal numbers of glial cells and neurons with 84.6 billion glia and 86.1 billion neurons.[1] The ratio differs between its different parts. The glia/neuron ratio in the cerebral cortex is 3.72 (60.84 billion glia; 16.34 billion neurons) while that of the cerebellum is only 0.23 (16.04 billion glia; 69.03 billion neurons). The ratio in the cerebral cortex gray matter is 1.48 (the white matter part has few neurons). The ratio of the basal ganglia, diencephalon and brainstem combined is 11.35.[1]

Most cerebral cortex glia are oligodendrocytes (75.6%) then astrocytes (17.3%) and least for microglia (6.5%)[17]

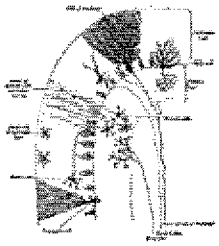
The amount of brain tissue that is made up of glia cells increases with brain size: the nematode brain contains only a few glia; a fruitfly's brain is 25% glia; that of a mouse, 65%; a human, 90%; and an elephant, 97%.[18]

Additional images

Oligodendrocyte

- 

Section of central canal of medulla spinalis, showing ependymal and neuroglial cells.

- 

Transverse section of a cerebellar folium.

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- Media related to Glia at Wikimedia Commons
- "The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease." (http://download.cell.com/images/edimages/neuron/pdf/barres.pdf) **Neuron** 60, November 6, 2008 by Ben Barres
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- New Source of Replacement Brain Cells Found (http://www.livescience.com/humanbiology/060816_neural_progenitors.html) - glial cells can transform into other cell types and reproduce indefinitely — tricks once thought exclusive to stem cells.
- Artist ADSkyler (http://www.anioman.com/Profile.php?viewedArtistID=1793201250&gallery=&page=1&back=1) (uses concepts of neuroscience and found inspiration from Glia)